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# REVEALING THE SECRETS OF MARCO: A TARGET FOR CANCER IMMUNOTHERAPY

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Cover illustration: Front: Taking a closer look at MARCO$^{+}$ macrophages. Back: MARCO$^{+}$ macrophages activate NK cells to increase their tumor cell killing. By Mitch Eisinger.


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# Revealing the secrets of MARCO: A target for cancer immunotherapy

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To my parents

Für meine Eltern

It always seems impossible until it`s done

- **Nelson Mandela**

# ABSTRACT

Our immune system protects us from infectious agents and sustains the homeostasis in our body. However, the immune system can also play a role in initiation and progression of diseases such as autoimmune diseases and cancer. Cancer is still one of the leading causes for death worldwide and inflammation became one of the hallmarks of cancer. Besides carrying out anti-tumor immune responses, the immune system also supports tumor growth through different aspects, such as promoting vascularization and suppressing other immune cells. Thus, targeting the immune system and harnessing its potential for anti-cancer therapy is a promising approach for treating cancer patients. Especially tumor-associated macrophages (TAMs) have shown to exert many tumor-supporting properties and represent a predominant immune cell population in most of the tumors. Therefore, the focus of this thesis was to evaluate the role of scavenger receptor MARCO on macrophages in immune responses and to reveal its potential as target for cancer immunotherapy.

**Paper I** identifies a novel interaction between marginal zone macrophages (MZMs) and marginal zone B cells (MZBs) that regulates antigen (Ag) transport into the follicle and Ag deposition onto follicular dendritic cells (FDCs) in the spleen. This can be modulated by targeting MARCO on MZMs by monoclonal antibodies (Abs) which leads to less Ag shuttling into the follicle, decreased Ag deposition to FDCs and to a subsequent reduced adaptive immune response. Anti-MARCO Abs can also be found in human system lupus erythematosus patients and this study may clarify the reason for their increased susceptibility for infections.

**Paper II** reveals MARCO as novel marker for a distinct tumor-promoting macrophage subtype in melanoma, breast and colon cancer mouse models. Anti-MARCO Ab treatment leads to reduced tumor growth and metastases and reprograms the $MARCO^+$ immune suppressive TAMs towards a pro-inflammatory phenotype. The combination of anti-MARCO Abs with checkpoint inhibitor anti-CTLA-4 shows enhanced efficacy and suggests MARCO targeted therapy as a promising cancer immunotherapy approach. Moreover, MARCO expressing TAMs can also be found in human breast cancer and melanoma patients.

**Paper III** investigates the mechanism of how anti-MARCO Ab treatment leads to reduced tumor growth in melanoma: Targeting MARCO by Abs leads to the activation of natural killer (NK) cells to increase their TRAIL-dependent tumor cell killing. $MARCO^+$ TAMs display a perivascular macrophage phenotype and targeting MARCO not only leads to metabolic re-programing of the $MARCO^+$ TAMs but results also in less confuse tumor vascularization. We confirmed enhanced efficacy of anti-MARCO Abs in combination with checkpoint inhibitors anti-PD-1 or anti-PD-L1 Abs and produced anti-human MARCO (hMARCO) Abs by immunizing mice with human MARCO protein to translate our findings to humans. Co-culture experiments with human $MARCO^+$ macrophages and T cells or NK cells validate that targeting hMARCO by Abs re-activates the effector cells leading to increased activation, proliferation and human melanoma cell killing *in vitro*.

**Paper IV** studies MARCO expression on immune suppressive myeloid cells in human pancreatic ductal adenocarcinoma patients, a cancer type with very poor prognosis. MARCO expression is induced by tumor-derived IL-10 and under hypoxic conditions not only found on immune suppressive TAMs but also on myeloid derived suppressor cells. Targeting $MARCO^{+}$ myeloid cells by anti-human MARCO Ab restores cytotoxic anti-tumor activity of T cells and NK cells including increased activation, proliferation and human pancreatic cancer cell killing *in vitro*.

Altogether, the work presented in this thesis gives us new insights into the biology of scavenger receptor MARCO, increases our understanding of the tumor microenvironment and how we can re-activate macrophages and modulate the immune suppressive tumor microenvironment by monoclonal Abs. Targeting MARCO enhances anti-tumor responses and the cytotoxicity of effector cells making it a promising approach for further immunotherapies. Our findings contribute to the design of new anti-cancer therapies including combining other immunotherapies with anti-MARCO Ab.

# ZUSAMMENFASSUNG

Das Immunsystem ist unser Abwehrsystem, welches uns vor Krankheitserregern und Infektionen schützt, das Gleichgewicht im Körper aufrecht erhält und somit Autoimmunerkrankungen und Gewebeschädigungen verhindert. Das Immunsystem besteht aus verschiedenen Immunzellarten, die auch als weiße Blutkörperchen bekannt sind. Sie kommunizieren miteinander und beschützen uns gemeinsam vor eindringenden Erregern und entarteten Zellen. Die Immunzellart, die für diese Thesis von besonderer Bedeutung ist, ist der Makrophage. Makrophagen sind Fresszellen, die Krankheitserreger direkt aufnehmen und zersetzen können oder aber andere Immunzellen durch die Produktion von Immunmodulatoren zum Ort der Entzündung rufen. Sie sind in allen Organen vorhanden und zirkulieren durch unsere Gefäße, um die Umgebung nach Fremdkörpern ab. Neben dem "Fressen" von Pathogenen, vernichten Makrophagen auch körpereigene Zellen, die zu alt sind, sterben oder Unregelmäßigkeiten aufweisen. Diese Zellen stellen ein potentielles Risiko dar und könnten durch Entartung zur Krebsentstehung beitragen.

Krebs ist noch immer eine der tödlichsten Krankheiten weltweit und es wurde bewiesen, dass eine Entzündung im Körper die Tumorentstehung begünstigen und das Immunsystem dabei sogar eine unterstützende Rolle spielen kann. Somit sind Immunzellen bei der Tumorbekämpfung ein zweiseitiges Schwert: Zum einen hat das Immunsystem eine anti-tumorale Wirkung. Am Anfang, wenn sich entartete Tumorzellen vermehren, werden unsere Abwehrzellen durch Immunmodulatoren gerufen, um den Tumor zu eliminieren. Diese Immunmodulatoren werden sowohl von gewebseigenen Zellen als Gefahrsignal als auch von Tumorzellen selbst ausgeschüttet und weisen den Immunzellen den Weg. So werden Abwehrzellen rekrutiert, die anfangs aktiv gegen die Tumorzellen vorgehen. Zum anderen sind Tumorzellen schlau und überlisten leider oft genug das Immunsystem, welches sie gezielt wissen "umzupoolen", damit es dem Tumor dann beim Wachstum hilft. Dies geschieht durch verschiedene Stoffe, die der Tumor ausschüttet um eine immun-unterdrückende Umgebung zu schaffen, welche die Immunzellen vom aktiven Bekämpfen der Tumorzellen abhält. Auch neu eintreffende Immunzellen werden so "ausgeschaltet" und der Tumor kann wachsen ohne vom Immunsystem bekämpft zu werden. Es ist sogar bekannt, dass Immunzellen den Krebszellen beim Metastasieren helfen und ihnen Zutritt zu den Lymph- und Blutgefäßen gewähren.

Da Immunzellen in so gut wie allen Tumorarten vorkommen und dabei aber leider meist tumor-unterstützend agieren, haben Wissenschaftler in den letzten Jahren daran gearbeitet, diese ausgeschalteten Abwehrzellen zu re-aktivieren, damit sie die Krebszellen erfolgreich bekämpfen können. Das hat den Wissenschaftlern James P. Allison und Tasuku Honjo im Jahr 2018 den Nobel Preis der Medizin gebracht. Sie waren die ersten, die herausgefunden haben, wie man die "Bremse" von einzelnen Immunzellen löst. Diese re-aktivierten Immunzellen können dann wieder verstärkt gegen Tumorzellen vorgehen, was dann zu einer Verkleinerung der Tumore und oft sogar zu kompletter Heilung führt. Leider sprechen nicht alle Patienten auf diese Art von Krebstherapie an, der Grund dafür ist bislang noch unbekannt. Deshalb arbeiten Wissenschaftler auf der ganzen Welt noch immer daran weitere Moleküle/Ziele auf

Immunzellen zu finden, welche man für die Krebs-Immuntherapie nutzen und eventuell auch in Kombination mit anderen Therapieansätzen anwenden kann, z.B. mit einer Chemotherapie.

Die hier vorliegende PhD Arbeit beschäftigt sich hauptsächlich mit tumor-assoziierten Makrophagen, welche häufig in großen Zahlen in Tumoren vorkommen, dabei meistens pro-tumoral sind und das Tumorwachstum unterstützen. In dieser Thesis untersuche ich die Rolle von dem Rezeptor MARCO, der auf der Außenseite von Makrophagen zu finden ist, in Immunantworten aber auch in Bezug auf Tumore. Die zentrale Frage dabei ist: Ist MARCO ein potenzielles Ziel für Krebs-Immuntherapien?

**Artikel I** beschäftigt sich mit der Interaktion zwischen den $MARCO^+$ Makrophagen und den B Zellen in der Milz. B Zellen sind die Immunzellen im Körper, die für die Antikörperproduktion zuständig sind und die Milz ist der Ort, an dem wichtige Prozesse für den Start einer Immunantwort stattfinden. Die Milz filtert das Blut des Körpers und die Immunzellen scannen es auf potenzielle Krankheitserreger, die sie dann aus dem Blut herausfangen, eliminieren und dann abhängig vom Pathogenen eine spezifische Immunreaktion einleiten. In unserer Studie haben wir herausgefunden, dass die Immunreaktion verändert wird, wenn man Mäusen Antikörper intravenös spritzt, die den Rezeptor MARCO spezifisch erkennen und binden. Wir haben die Interaktion zwischen den $MARCO^+$ Makrophagen und den B Zellen in der Milz so modifiziert, dass die B Zellen weniger Antigen transportieren und im Endeffekt auch weniger spezifische Antikörper herstellen. Patienten, die an der Autoimmunerkrankung systemischer Lupus erythematodes leiden, haben auch Antikörper gegen MARCO in ihrer Blutlaufbahn, von denen man aber nicht genau weiß was ihre Aufgabe ist. Unsere Ergebnisse könnten eine Erklärung für ihr erhöhtes Infektionsrisiko sein.

In **Artikel II** zeigen wir zum ersten Mal in drei verschiedenen Maus-Tumormodellen, dass MARCO ein Marker für die pro-tumoralen und immun-unterdrückenden tumor-assoziierten Makrophagen ist: Genauer gesagt in Brustkrebs, Hautkrebs und Darmkrebs. Wenn wir diese Mäuse mit anti-MARCO Antikörpern behandeln, haben sie kleinere Tumore und auch weniger Metastasen in der Lunge. Mithilfe der Antikörper-Behandlung können wir diese pro-tumoralen Makrophagen um-programmieren und re-aktivieren, um die Krebszellen zu bekämpfen. Auch die Kombination von unseren anti-MARCO Antikörpern und den bereits in Menschen genutzten Immuntherapie, welche auf dem Molekül CTLA-4 basiert und von Nobelpreis-Träger James P. Allison entdeckt wurde, ist vielversprechend: In der Kombination sehen wir noch kleinere Tumore verglichen zu den einzelnen Behandlungen. Um unsere Entdeckung in Zukunft auch beim Menschen anwenden zu können, wollten wir wissen, inwiefern MARCO in humanen Tumoren verbreitet ist. Tatsächlich haben wir MARCO auf pro-tumoralen Makrophagen in mehreren humanen Tumorarten gefunden, z.B. in Brustkrebs und Hautkrebs. Deshalb glauben wir, dass eine anti-MARCO gerichtete Therapie eine wirklich vielversprechende Art der Krebs-Immuntherapie ist.

In **Artikel III** beschäftigen wir uns mit dem Mechanismus, warum das Spritzen von anti-MARCO Antikörpern zu kleineren Tumoren führt. Wie funktioniert das Ganze? Was genau passiert in den Makrophagen? Und sind die Makrophagen alleine für die Tumorreduktion

verantwortlich? In diesem Artikel beschäftigen wir uns nur mit Hautkrebs als Modell und sehen, dass die Umprogrammierung der Makrophagen viele intrazelluläre Prozesse beinflusst und auch den kompletten metabolischen Haushalt der Zelle verändert. Interessanterweise re-programmiert die auf MARCO zielende Immuntherapie nicht nur die Makrophagen, sondern aktiviert auch andere Immunzellen im Tumor, die natürliche Killerzellen (NK Zellen) genannt werden. Diese NK Zellen können Krebszellen direkt töten, indem sie mit ihnen in Kontakt treten und mithilfe von Rezeptoren-Verbindungen den Tod der Tumorzelle induzieren oder aber ”Killer-Substanzen” ausstoßen, die die Tumorzelle vernichten. In diesem Artikel kombinieren wir anti-MARCO Antikörper mit anderen Immuntherapie-Antikörpern, die auf die Moleküle PD-1 und PD-L1 gerichtet sind und sehen erneut, dass eine Kombination hervorragende Ergebnisse zeigt und wir noch kleinere Tumore bekommen als in den Einzelbehandlungen. Ein weiterer wichtiger Bestandteil dieser Studie ist, dass wir Antikörper spezifisch für den menschlichen MARCO Rezeptor statt dem Maus Rezeptor hergestellt haben. Somit können wir nun auch menschliche Zellen erreichen und sehen in *in vitro* Experimenten in der Petrischale, dass die Behandlung von menschlichen $MARCO^{+}$ Makrophagen mit dem Antikörper ebenfalls zur Umprogrammierung der Makrophagen zu einem anti-tumoralen Status führt, so wie in den Mäusen. Für diese Experimente in der Petrischale werden die Zellen in sogenannten tumor-konditioniertem Medium kultiviert. Dies ist das Medium, in dem vorher echte Krebszellen gehalten wurden und somit alle Stoffe enthält, die sonst auch im wirklichen Tumor im Körper von den Krebszellen ausgeschüttet werden. Dieses System dient dazu, die Tumorkonditionen bestmöglich und so realitätsgetreu wie möglich zu simulieren. In den Petrischalen-Experimenten sehen wir, dass die einzelnen Immunzellen unterdrückt und ausgeschaltet werden. Wenn wir die Makrophagen vorher aber mit unseren anti-humanen MARCO Antikörpern behandeln, können wir wichtige Immunzellen retten und wieder ihre anti-tumor Aktivität und Tötungskapazität erhöhen. Eine dieser Immunzellen sind die bereits oben genannten NK Zellen.
In **Artikel IV** beschäftigen wir uns mehr mit MARCO im Menschen und fokussieren uns hier auf Pankreaskrebs, einer der schlimmsten, schwer heilbaren Krebsarten. Auch dort finden wir MARCO auf pro-tumoralen Makrophagen im Tumor und können in der Petrischale zeigen, dass auch im Bezug auf Pankreaskrebs, die Behandlung der Makrophagen mit den anti-MARCO Antikörpern zu erhöhtem Töten von Tumorzellen und vermehrter Aktivität von T Zellen und NK Zellen führt.

Zusammenfassend kann man sagen, dass diese Arbeit Einsicht in die Biologie von dem Rezeptor MARCO gibt und wir nun wissen, dass er als immuntherapeutisches Ziel geeignet ist. Mithilfe von auf MARCO gerichteten Antikörpern können wir den Tumor modulieren und die dort vorhandenen Immunzellen re-programmieren, die vorher vom Tumor unterdrückt wurden. So erzielen wir eine Reduktion des Tumors und re-aktivieren die Abwehrzellen , die ihrer Aufgabe wieder nachgehen und den Krebs bekämpfen. Unsere Ergebnisse sind vielversprechend und helfen dabei, klinische Studien zu planen und anti-MARCO Antikörper herzustellen, die dann eventuell in Kombination mit anderen Therapiemöglichkeiten an Krebspatienten angewendet werden können.

# LIST OF SCIENTIFIC PAPERS

I. Kajsa E. Prokopec, **Silke Sohn***, Anna-Maria Georgoudaki*, Fredrik Wermeling, Hans Grönlund, Emma Lindh, Michael C. Carroll, Mikael C. I. Karlsson
(* equal contribution)
**Marginal zone macrophages regulate antigen transport by B cells to the follicle in the spleen via CD21**
*The Journal of Immunology, 2016 Sept 15, 197(6):2063-8*

II. Anna-Maria Georgoudaki, Kajsa Prokopec, Vanessa F. Boura, Eva Hellqvist, **Silke Sohn**, Jeannette Östling, Robert A. Harris, Mattias Rantalainen, Daniel Klevebring, Malin Sund, Jonas Fuxe, Charlotte Rolny, Fubin Li, Jeffrey V. Ravetch, Mikael C.I.Karlsson
**Reprogramming tumor-associated macrophages by antibody targeting inhibits cancer progression and metastasis**
*Cell Reports, 2016 May 31, 15(9), 2000-2011*

III. **Silke Eisinger**, Dhifaf Sarhan, Vanessa F. Boura, Sofia Tyystjärvi, Ganna Oliynyk, Marie Arsenian-Henriksson, David Lane, Stina Wickström, Rolf Kiessling, Dagmara Kaczynska, Shigeaki Kanatani, Evangelia Daskalakis, Craig Wheelock, Saikiran Sedimbi, Benedict Chambers, Jeffrey V. Ravetch, Mikael C.I. Karlsson
**Targeting scavenger receptor MARCO on tumor-associated macrophages activates TRAIL- dependent tumor cell killing by NK cells in melanoma**
*Manuscript*

IV. Dhifaf Sarhan, **Silke Eisinger**, Caroline Driescher, Giorgia Palano, Shuijie LI, Susanne Schlisio, Sarah Schott, Patrick Smith, Jeffrey V. Ravetch, Rainer Heuchel, Matthias Löhr, Mikael C. I. Karlsson
**Antibody targeting of tumor-associated macrophages in pancreatic cancer remodels the tumor microenvironment and revives immune targeting of tumor cells**
*Manuscript*

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| **Ab** | Antibody |
| **ADCC** | Antibody- dependent cell cytotoxicity |
| **Ag** | Antigen |
| **AID** | Activation-induced cytidine deaminase |
| **AMP** | Adenosine monophosphate |
| **ANG2** | Angiopoietin 2 |
| **APCs** | Antigen presenting cells |
| **Arg-1** | Arginase-1 |
| **ATP** | Adenosine triphosphate |
| **BCR** | B cell receptor |
| **BMDM** | Bone marrow-derived macrophages |
| **CAR** | Chimeric antigen receptor |
| **CCL** | Chemokine (C-C motif) ligand |
| **CCR** | Chemokine (C-C motif) receptor |
| **CD** | Cluster of differentiation |
| **CD40L** | CD40 ligand |
| **CDR** | Complementarity determining region |
| **CLP** | Common lymphoid progenitor |
| **CSF-1(R)** | Colony stimulating factor-1 (receptor) |
| **CTLA-4** | Cytotoxic T-lymphocyte antigen-4 |
| **CXCL** | CXC chemokine ligand |
| **CXCR** | CXC chemokine receptor |
| **DAMP** | Danger-associated molecular pattern molecule |
| **DC** | Dendritic cell |
| **Dim** | Diminished |
| **DR4/5** | Death receptor 4/5 |
| **ECM** | Extracellular matrix |
| **EMT** | Epithelial- mesenchymal transition |
| **Fc** | Fragment, crystallizable |
| **FcγR** | Fc gamma receptor |
| **FDA** | The Food and Drug Administration |
| **FDC** | Follicular dendritic cell |
| **FOB** | Follicular B cell |
| **FoxP3** | Forkhead box P3 |
| **GC** | Germinal center |
| **GM-CSF** | Granulocyte-macrophage colony-stimulating factor |
| **HCC** | Hepatocellular carcinoma |

| | |
|---|---|
| **HIF-1α** | Hypoxia-inducible factor 1-alpha |
| **HLA** | Human leukocyte antigen |
| **hMARCO** | Human scavenger receptor MARCO |
| **HNSCC** | Head and neck squamous cell carcinoma |
| **HSC** | Hematopoietic stem cell |
| **IFN-γ** | Interferon- gamma |
| **Ig** | Immunoglobulin |
| **IL** | Interleukin |
| **ILC** | Innate lymphoid cell |
| **IMC** | Immature myeloid cell |
| **iNOS** | Inducible nitric oxide synthase |
| **i.p.** | Intraperitoneally |
| **i.v.** | Intravenously |
| **IRF** | Interferon regulatory factor |
| **ITAM** | Immunoreceptor tyrosine-based activation motif |
| **ITIM** | Immunoreceptor tyrosine-based inhibitory motif |
| **IVIG** | Intravenous immunoglobulin |
| **KIR** | Killer cell immunoglobulin-like receptor |
| **LDL** | Low- density lipoprotein |
| **LPS** | Lipopolysaccharide |
| **LTi** | Lymphoid tissue inducer |
| **MARCO** | Macrophage receptor with collagenous structure |
| **MCM** | Medullary cord macrophage |
| **M-CSF** | Macrophage colony-stimulating factor |
| **MDSC** | Myeloid- derived suppressor cell |
| **MHC-I/II** | Major histocompatibility complex – I/II |
| **MM** | Metallophilic macrophage |
| **M-MDSC** | Monocytic myeloid-derived suppressor cell |
| **MMP** | Matrix metalloproteinases |
| **MSM** | Medullary sinus macrophage |
| **MyD88** | Myeloid differentiation primary response 88 |
| **MZ** | Marginal zone |
| **MZB** | Marginal zone B cells |
| **MZM** | Marginal zone macrophage |
| **NALP3** | NACHT, LRR and PYD domains-containing protein 3 |
| **NF- κB** | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| **NK(T) cells** | Natural killer (T) cells |
| **NLRP3** | NACHT, LRR and PYD domains-containing protein 3 |
| **NO** | Nitric oxide |

| | |
|---|---|
| **NOD2** | Nucleotide-binding oligomerization domain-containing protein 2 |
| **OXPHOS** | Oxidative phosphorylation |
| **PAMP** | Pathogen-associated molecular pattern molecule |
| **PBMC** | Peripheral blood mononuclear cell |
| **PDAC** | Pancreatic ductal adenocarcinoma |
| **PD-(L)1** | Programmed cell death protein (ligand)- 1 |
| **PI3K** | Phosphoinositide 3-kinase |
| **PMN-MDSC** | Polymorphonuclear myeloid-derived suppressor cell |
| **PRR** | Pattern recognition receptor |
| **ROS** | Reactive oxygen species |
| **SIGN-RI** | specific intracellular adhesion molecule-grabbing nonintegrin R1 |
| **SIRP-α** | Signal regulatory protein- α |
| **SLE** | Systemic lupus erythematosus |
| **SR** | Scavenger receptor |
| **SRCR** | Scavenger receptor cysteine-rich domain |
| **SSM** | Subcapsular sinus macrophage |
| **STAT** | Signal transducer and activator of transcription |
| **T1/2** | Transitional 1/2 |
| **TAM** | Tumor-associated macrophage |
| **TCGA** | The Cancer Genome Atlas |
| **TD** | Thymus- dependent |
| **TEM** | TIE2- expressing monocytes/macrophages |
| **$T_{FH}$ cell** | Follicular T helper cell |
| **TGF-β** | Transforming growth factor-beta |
| **$T_H$ cell** | Helper T cell |
| **TI** | Thymus-independent |
| **TIE2** | Angiopoietin-1 receptor |
| **TIGIT** | T cell immunoreceptor with Ig and ITIM domains |
| **TME** | Tumor microenvironment |
| **TMEM** | Tumor microenvironment of metastasis |
| **TNF** | Tumor necrosis factor |
| **TRAIL** | Tumor necrosis factor–related apoptosis-inducing ligand |
| **$T_{reg}$ cell** | Regulatory T cell |
| **WT** | Wildtype |

# 1 INTRODUCTION

## 1.1 THE IMMUNE SYSTEM

The immune system is the host`s line of defense against infectious agents and an important player in maintaining tissue homeostasis. Its development starts during embryogenesis and until birth it has evolved as a sophisticated network of effector cells and molecules that still develops during life and discriminates between self and non-self.

Some of the first experiments in the field of immunology go back to Edward Jenner who protected a young man against smallpox by administration of cowpox virus in 1796. This was the first proof of immunological memory and the first step into the field of vaccinations. Another pioneering finding was discovered by Ilya Metchnikoff in 1866 when he discovered leukocytes were able to migrate to the site of infection, engulfing and digesting pathogens. He named the cells "phagocytes" and the process phagocytosis [1] which we today know as a part of the unspecific and quickly responding innate immunity. Metchnikoff was awarded the Nobel Prize for Physiology or Medicine in 1908 as his discovery was the birth of cellular immunity.

In the late 19$^{th}$ century, more groundbreaking discoveries were made: In 1884, Robert Koch identified that specific microorganisms are responsible for certain infectious diseases, including tuberculosis. Furthermore, Louis Pasteur was inspired by Edward Jenner and discovered a vaccine against chicken cholera in the 1880s realizing that old bacteria are not as deadly and can be used for immunization. Paul Ehrlich was one of the first to postulate that antitoxins, today known as antibodies (Abs), are produced to battle bacteria and bind bacterial toxins. In the 1890s, Emil von Behring and Shibasaburo Kitasato discovered that the serum of animals contained soluble factors that neutralize toxins released by bacteria. Besides studies dealing with soluble Abs and cellular immunity, Jules Bordet discovered the complement system in 1899 as she detected that heat-stable antitoxins as well as heat-sensitive components in the serum were responsible for the disruption of bacterial cell membranes and they act together with Abs to kill pathogens.

As we know today, the immune system consists of two branches, the innate and the adaptive immune system – a sophisticated network of highly specialized immune cells which collaborate to protect the host from infection and maintaining tissue homeostasis. Here, the two branches will be discussed and the cells and mechanisms most important for this thesis are emphasized.

### 1.1.1 Lymphoid organs

The development of most immune cells starts in the bone marrow where leukocytes emerge from hematopoietic stem cells (HSC) in the process of "hematopoiesis" before they differentiate further into lymphoid or myeloid lineage cells. Besides that, cells of the myeloid lineage can also originate in the yolk sac or fetal liver during embryogenesis. There are central or primary lymphoid organs where lymphocytes are produced and peripheral or secondary lymphoid organs where naïve lymphocytes are maintained and antigens are presented to them, thus adaptive immune responses are initiated.

The central or primary lymphoid organs are the bone marrow (where B cells develop) and thymus (the site where T cells arise from); the peripheral or secondary lymphoid organs include spleen and lymph nodes. Sometimes people also refer to tertiary lymphoid tissues which include Peyer`s patches, mucosa/gut- associated lymphoid tissues (MALT/GALT) and tonsils. These are the tissues where immune cells perform immunoregulatory functions. To move through the body and to distant sites, immune cells travel through blood vessels and specialized vessels called lymphatics.

The spleen is the biggest peripheral lymphoid organ and together with lymph nodes these are the sites where immune cells have cell-to-cell contact to start immune responses and there are B cells, T cells and monocytes present. The spleen is divided into red pulp and white pulp: In the red pulp there are mainly red pulp macrophages screening for pathogens and removing damaged and aged red blood cells. The white pulp is the site where innate meet adaptive immune cells to initiate immune responses. It consists of strictly determined structures including T cell and B cell follicles. In the latter, the antigen (Ag) specific adaptive immune response arises, and germinal centers (GC) are formed. In rodents, the lymphoid white pulp and the scavenging red pulp is separated by the marginal zone (MZ) (Figure 1). The MZ is critical for capturing blood-borne pathogens by highly specialized phagocytes, such as marginal zone macrophages (MZMs), metallophilic macrophages (MMs) and dendritic cells (DCs). These phagocytes express a variety of pattern recognition receptors (PRR) to detect a wide spectrum of microbial antigens. MZMs are crucial players in this process [2] and align the outside of the MZ. Next to the highly phagocytic MZMs, MMs are located on the inside of the MZ [3]. Besides macrophages, there is a distinct B cell type present in the MZ, called the marginal zone B cells (MZBs). MZBs are located strategically well and can respond quickly initiating T cell independent immune responses. Inside the follicular (FO) area of spleen and also lymph nodes there is a B cell subset that it different than the MZB called FO B cells (FOBs). FOBs represent the majority of B cells in the spleen [4,5] and are the ones that are involved in T- cell – dependent Ab responses. MZBs are known to travel between the MZ and follicle transporting captured Ag into the follicles and deposit Ag on follicular dendritic cells (FDCs) [6–9]. Furthermore, it has been shown that the interaction between MZMs and MZBs is required to retain the MZ structure [10]. In chapter 1.2.2 the interdependence of MZMs and MZBs are described in more details and paper I in this thesis thematizes how the crosstalk between MZMs and MZBs can be modulated.

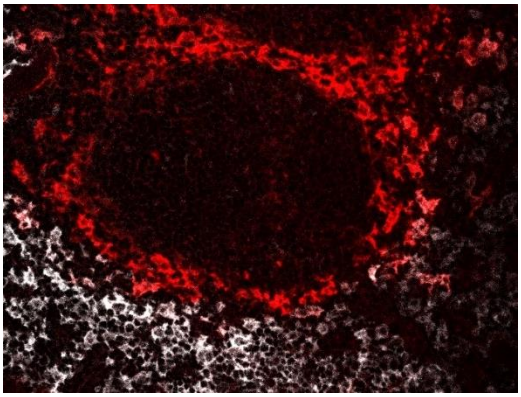

**Figure 1: Structure of the marginal zone in the spleen.** MARCO on marginal zone macrophages (red), F4/80 on red pulp macrophages (white).

### 1.1.2 Innate immune system

The innate immune system is the first line of defense against infectious agents and invading microorganisms which is already functional at birth and has been found in invertebrates and vertebrates. The innate immune system consists of physical barriers such as skin and mucosa, but also of anti-microbial plasma proteins (complement) and innate immune cells. The latter are mostly myeloid cells, including monocytes, macrophages, DCs and granulocytes (neutrophils, basophils, eosinophils, mast cells) but also include natural killer (NK) cells and innate lymphoid cells (ILC) that have lymphoid progenitor cells. The innate immune response is also called "unspecific immunity" as it acts rapidly and distinguishes between "self" and "non-self" based on widely conserved domains that are expressed on pathogens but differentiate host cells. These structures are called danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) or double stranded RNA [11] and are recognized by a variety of PRRs. PRRs can be on the cell surface such as scavenger receptors, Toll-like receptors (TLR), c-type lectin receptors and complement receptors or intracellularly, such as retinoic acid- inducible gene (RIG)-I-like receptors (RLR), NOD-like receptors. Ligand binding by PRRs can result in the internalization of the receptor for further degradation mediating a tailored immune response [12,13]. Extracellular adenosine triphosphate (ATP) is also known as a danger- signal that can activate immune cells, polarizes macrophages towards an inflammatory state and leads to inflammation [14,15]. Extracellular ATP can be sensed by ATP-sensing receptors, such as P2X purinoceptor 7 (P2X7R) which leads to intracellular signaling and activation of the immune cells [16,17] or it is broken down to adenosine monophosphate (AMP) by ectonucleotidase CD39 and further processed to adenosine by CD73 which is known for its immunomodulatory effects [18]. Adenosine can suppress T cell proliferation, the release of pro-inflammatory cytokines and cytotoxic activity by NK cells [19,20]. Upon the detection of pathogens, innate immune cells take action against them and some travel to peripheral or secondary lymphoid organs initiating adaptive immunity resulting in the eradication of the intruder.

### 1.1.3 Adaptive immune system

In contrast, the second branch of the immune system is the adaptive immune system that includes humoral and cell-mediated immunity. It ensures Ag-specific immune responses as well as memory and is acquired during life. Adaptive immune responses have been only found in vertebrates. B and T lymphocytes are the players of the adaptive immune system and in contrast to innate immune responses, they need to encounter the Ag they are specific for to be activated and able to combat it. Thus, the initiation of an adaptive immune responses takes more time compared to innate immunity but also has a memory which innate immune cells do not provide. While B cells complete their development in the bone marrow, T cell precursors migrate to the thymus for their maturation. Adaptive immune responses provide immunity against most pathogens faced during a lifetime. In adults, most of the B cells still arise from bone marrow precursors whereas T cell production in the thymus is decreasing and T cell numbers are maintained by the division in the periphery. Upon Ag recognition, B and T cells undergo rapid clonal expansion and selection processes to avoid auto-reactive cells. It depends

on the Ag which kind of T cells will be activated: different subtypes of T helper ($T_H$) cells to suppress or modulate the immune response or cytotoxic T cells for the killing of infected and aberrant cells. Upon activation, B cells undergo affinity maturation and are responsible to produce Ag-specific Abs.

## 1.2 LYMPHOID CELLS

Arising from HSC during hematopoiesis in the bone marrow, lymphoid progenitors give rise to B cells, T cells, NK cells and innate ILCs but also γδ T cells and NKT cells. As important cell types for this thesis, only T cells, B cells and NK cells will be further described here.

### 1.2.1 T cells

T cells arise from the common lymphoid progenitor (CLP) and belong to the adaptive immune system. Precursor cells migrate to the thymus to become mature T cells which express a unique T cell receptor (TCR) that recognizes peptides in complex with major histocompatibility complex (MHC) on the surface of antigen presenting cells (APCs). The TCR consists of two glycoprotein chains, most of the T cells will become αβ T cells, whereas only around 5% will become γδ T cells. Besides TCRs, T cells express one of the co-receptors CD4 or CD8 which mostly determines which kind of antigens the cell will recognize: Ags presented in MHC-I complexes activate $CD8^+$ T cells (endogenous agents), MHC-II Ag complexes will activate $CD4^+$ T cells (exogenous agents). γδ T cells can mostly be found in epithelial and mucosal sites where they reside and do not express CD4 or CD8. However, it remains unknown which ligands γδ T cells have but they are thought to be not restricted to MHC molecules. Before T cells are released to the circulation, they undergo several maturation steps to ensure recognition of peptides in complex with self- MHC molecules (positive selection) but also to avoid self-reactive T cells that interact strongly with self-molecules by depletion as they undergo apoptosis (negative selection). The remaining cells are mature naïve T cells which enter the circulation and secondary lymphoid organs to encounter their specific Ag presented by APCs. Activation of $CD4^+$ and $CD8^+$ T cells always requires APCs that present the Ag in form of a peptide-MHC complex to T cells. DCs are the most professional and potent APCs but also other cells, such as B cells or macrophages can present Ag to T cells.

After activation, T cells undergo clonal expansion and differentiate into effector T cells dependent on the nature of the Ag. These effector T cells either migrate to the site of infection to kill infected or malignant cells or activate macrophages. $CD8^+$ T cells are appreciated as cytotoxic T cells that remove target cells. However, T cells can also travel to B cell areas to provide help to B cells for a proper humoral immune response (follicular $T_h$ cells). $CD4^+$ T cells are considered as $T_h$ cells and can further be divided into $T_h1$, $T_h2$, $T_h9$, $T_h17$, $T_h22$ and T regulatory ($T_{reg}$) subsets each with a unique repertoire of cytokine production [21,22]. $T_h1$ and $T_h17$ cells are known to support inflammation by the production of interferon gamma (IFN-γ) and thus activate cytotoxic cells, such as macrophages, NK cells and cytotoxic $CD8^+$ T cells. Furthermore, $T_h17$ cells produce cytokines, such as IL-17 and IL-21 and thus, support an inflammatory response. IL-17 can induce pro-inflammatory cytokine production and

recruitment of neutrophils, IL-21 stimulates the proliferation of $CD8^+$ T cells and induces NK cells activity and increases their cytotoxicity [21,23]. $T_h2$ cells help B cells to implement a humoral immune response and to differentiate into Ab producing plasma cells by secreting IL-4 and IL-10 amongst other cytokines. $T_{reg}$ cells are a more immune suppressive T cell subset, defined by the expression of CD25 and transcription factor FoxP3 and they are known to express IL-10 and TGF-β [24]. $T_h22$ cells express IL-22 and are mainly suggested to be involved in the pathogenesis of skin inflammatory diseases [22]. The most recently defined $T_h$ cell subset are $T_h9$ cells which have been so far classified as $T_h2$ cells but recently got classified as IL-9 producing $T_h9$ cell. They arise upon IL-4 and TGF-β stimulation and their main cytokine producing is IL-9, but they have also been shown to secrete IL-10 and IL-21 [21,25]. IL-9 has many effects on several immune cells, such as supporting immune suppressive functions of $T_{regs}$ or promoting $T_h17$ proliferation and it has been demonstrated to be inflammatory (promotes allergic airway inflammation) as well as anti-inflammatory (experimental autoimmune encephalomyelitis) so there is still a need of uncovering the nature of IL-9 and its effect on the immune system [26].

Cytotoxic $CD8^+$ T cells can also be activated through the interaction with other immune cells. Activated NK cells produce IFN-γ which mediates DCs to secrete IL-12 that further enhances $CD8^+$ T cells response and tumor killing [27]. Moreover, the re-polarization of TAMs towards a pro-inflammatory phenotype using cryo-thermal therapy in B16 melanoma resulted in the generation of cytotoxic $CD8^+$ T cells, supporting anti-tumor $CD4^+$ T cell activation and anti-tumor response [28]. Thus, the interaction with other immune cells is needed for potent T cell activation.

### 1.2.2 B cells

B cells belong to the adaptive immune system and are a distinct and unique cell type as they are the only Ab-producing cells of the immune system. Other functions are Ag presentation, cytokine production and they are involved in the generation of immunological memory. In 1890, the existence of antitoxins in the serum of animals has been discovered to provide immunity against diphteria and tetanus [29]. However, the discovery of the cells producing these antitoxins was only in 1965/66 by Max Cooper and Robert Good when they used chickens as model system and found that cells from the bursa of Fabricus (equivalent to the bone marrow) are responsible for antibody production (B cells) [30,31].

B cells develop from CLPs in the bone marrow where they go through several developmental stages: from CLP to early pro-B cells, late pro-B cells, pre-B cell to immature B cells. During that time B cells encounter as many Ags as possible to develop their unique B cell receptor (BCR) that is highly Ag specific. The BCR is a surface-bound immunoglobulin (Ig) and consists of a heavy and a light chain that binds to the Ag through its complementarity determining regions (CDRs). To provide a big repertoire of different BCRs, B cells undergo random gene rearrangement of their immunoglobulin (Ig) gene segments. A lot of B cells will be auto-reactive and will undergo receptor editing or clonal deletion – these are the mechanisms to protect the host against auto-reactive immune cells and is called “central tolerance”. All this

results in a plethora of many different BCRs (up to $10^8$ distinct receptors in the repertoire are possible) and thus, providing the selectivity and specificity of the adaptive immune system.

Non self-reactive B cells leave the bone marrow as immature transitional 1 (T1) B cells, migrate to splenic B cell follicles where they mature to T2 B cells. T1 B cells are characterized by their IgM expression, once they mature they become T2 B cells and gain IgD surface expression [32]. Furthermore, T1 B cells do not express CD21 (complement receptor 2) yet, whereas T2 B cells do express CD21 [24].

Mature B cells can then be divided into B-1 and B-2 cells [33,34]. In rodents, B-1 cells can mainly be found in the pleural and peritoneal cavities and are also referred to as innate-like B cells. $CD5^+$ B-1 cells develop already from fetal liver hematopoiesis and are long-lived self-renewing cells [33]. Murine B-1 cells have been shown to be the main producers of natural Ab pool. These are spontaneously secreted IgM Ab that are also expressed in steady-state conditions to act on pathogens quickly [35]. In contrast, B-2 cells become follicular (FO) or MZ B cells. FOBs are the most occurring B cell type in secondary lymphoid organs and they are homing to B cell follicles close to the T cell areas [33]. Upon T cell help, naïve FOBs undergo proliferation and can differentiate into plasma cells or memory B cells. In contrast, MZBs are self-renewing and non-circulating B cells resident in the splenic MZ. They are strategically well located to capture blood – borne pathogens in proximity to the APCs MZMs and DCs. MZBs are known to initiate rapid T-independent (TI) responses and are characterized by the expression of CD21 and CD1d. CD21 plays a crucial role in shuttling antigens to the follicle and CD1d is required to interact with natural killer T (NKT) cells by presenting lipid Ags [4,33,34]. MZBs are known to receive Ag from MZMs [4,36] and MZBs transport Ag into the follicle for deposition on FDCs [6,37]. MZBs regulate the capture capacity of MZMs as the depletion of MZBs results in less Ag capture by MZMs and less SIGN-$RI^+$ MZMs which in turn also affects the Ag capturing by B cells [38]. The interaction between MZBs and MZMs is important for the trafficking of MZBs to the follicle [10]. The interdependence of MZMs and MZBs is also validated by the fact that the loss of B cells led to the loss of MZMs and vice versa the loss of MZMs affected MZBs numbers [10,39]; furthermore, MZMs have been shown to be responsible for the retention of MZB [10].

The activation of B cells depends on the nature of the Ag: Upon encountering their specific Ag, B cells get activated and differentiate into Ab producing plasma- or memory B cells that are ultimately required for the humoral immunity. Non-protein Ags, such as lipids, do not need T cell involvement for activation and Ab production by B cells and are referred to as thymus-independent (TI) Ags. In contrast, there are protein Ags that do require the interaction of T and B cells; thus, these are called thymus-dependent (TD) antigens. TI immune responses can be further divided into type I and type II Ags. Type I Ags stimulate B cells through TLRs, whereas type II Ags are usually large polysaccharides with multiple antigenic binding sites crosslinking BCRs and thus activating B cells. TI Ags usually result in low affinity, mostly IgM Ab production and the response is short-lived compared to TD responses. In contrast, TD B cell activation requires potent T cell help. In lymphoid organs, naïve $CD4^+$ T cells recognize Ags

presented by APCs in the T cell area. At the same time, B cells encounter the same pathogen with their BCR which gets them activated and induces them to move towards the interface between B and T cells zone in lymphoid organs. B cells that get activated through BCR engagement also upregulate co-stimulatory molecules, internalize the Ag, processing and presenting it in the MHC-II molecule. The $CD4^+$ T cells in turn recognize the antigen-MHC-II complexes and further binding through co-stimulatory molecules activates the T cells to proliferate. These T cells are also called T follicular helper cells ($T_{FH}$ cells) and upon activation they upregulate CD40 ligand (CD40L) which binds CD40 on B cells and produce cytokines that promote B cell proliferation and differentiation into Ab-producing cells. At this stage, $T_{FH}$ cells determine the required Ab isotype by supporting isotype switching through cytokine production (for instance, IL-4 for IgG1 Ab, IFN-γ for IgG2a, TGF-β for IgG2b). The Ab isotype determines the Fc receptor specificity on effector cells leading to different function of immune cells. The proliferation of B cells in response to a TD Ag is called germinal center formation (GC) and occurs in the interface between B and T cell follicles in secondary lymphoid organs. Besides proliferating B cells, FDCs can be found in the GC that support B cell proliferation in the dark zone of the GCs before B cells migrate to the light zone where they undergo affinity maturation, needing help from FDCs and $T_{FH}$ cells. Here, long-lived plasma cells and memory cells are created. Through somatic hypermutation, mediated by the gene activation-induced deaminase (AID) which is also responsible for isotype class switching [40], B cells are going through the process of affinity maturation and only the B cells with Ab of the highest Ag affinity are selected and survive. These activated B cells then differentiate into plasma- or memory cells that can be found in extrafollicular areas in lymphoid organs or they migrate back to the bone marrow where they reside.

Besides their primary role in humoral immune responses, B cells also have other functions, such as antigen presentation [41], the production of immunomodulatory cytokines, including IL-2, IL-4, IL-6, IFN-γ, IL-12 or TNF- α and the regulatory B cell ($B_{reg}$) subtype that is known to produce the immune suppressive molecules IL-10 and TGF- β [42] .

Auto-reactive B cells play a crucial role in many autoimmune diseases, such as Systemic lupus erythematosus (SLE) where B cells have been identified as the main players [43]. SLE can affect every organ and autoantibodies are the hallmark of this disease, such as anti-DNA or anti-MARCO autoantibodies [43,44].

### 1.2.3 Innate lymphoid cells and natural killer cells

Natural killer (NK) cells were discovered in 1975 simultaneously by Rolf Kiessling, Eva Klein and Hans Wigzell at Karolinska Institutet in Stockholm [45,46] and Herberman et al. at the University in Pittsburgh [47,48]. They were described as a subset of lymphocytes that can spontaneously kill tumor cells without any pre-stimulation and in 1986 Klas Kärre demonstrated their killing mechanism by proposing the ”missing-self” theory [49,50]. In mice, NK cells are characterized as $NK1.1^+$ $NKp46^+$ $CD3^-$ cells; in humans, NK cells are mostly divided into two subsets $CD56^{dim}$ $CD16^+$ and $CD56^{bright}$ $CD16^{dim/-}$. The $CD56^{dim}$ $CD16^+$ population constitutes 90% of all NK cells, is mostly present in the circulation and shows enhanced

cytolytic and killing capacity compared to $CD56^{bright}$ $CD16^{dim/-}$ NK cells which are the cytokine producing subtype [51]. NK cells were always counted as the third largest lymphocyte population besides B and T cells. However, recently they got re-categorized and classified as a member of the innate lymphoid cells (ILCs). The group of ILCs counts five members: NK cells, ILC1, ILC2, ILC3 and the lymphoid tissue inducer cells [52].

NK cells express a mixture of activating and inhibitory receptors that are important for their cytolytic function. As the "missing-self" theory states, NK cells recognize self-MHC complexes on the surface of cells which leads to their inhibition through the inhibitory killer cell Ig-like receptors (KIRs) in humans and the Ly49 receptor family in mice. During their development, NK cells have been educated to recognize a plethora of self MHC-I – antigen complexes. However, when the cognate self-MHC-I molecules are missing because pathogens or tumor cells abrogate its expression as an immune escape route, NK cells get activated and kill this cell [49,50]. But NK cells can also kill by ligation of their activating receptors, such as DNAM1, NKp44, NKp46, NKG2D whose ligands are expressed on malignant or infected cells. Therefore, the activation state of NK cells is highly dependent on the balance of inhibitory and activating signals.

NK cells store cytotoxic granules in their cytoplasm containing effector molecules, such as the pore-forming molecule perforin or protease granzyme B which are released upon activation. Together they lead to pore formation in the membrane of target cells and subsequently to their death. NK cell killing mechanisms also comprise receptors that are mediating target cell killing, such as Fas ligand or tumor necrosis factor- related apoptosis-inducing ligand (TRAIL). Its ligands death receptors 4 (DR4) and DR5 belong to the TNF superfamily "death" receptors and are expressed on many cells including tumor cells. Upon binding, TRAIL stimulates DR4/DR5 expressing cells to undergo apoptosis in a caspase – 8 dependent matter. Another receptor – dependent way of killing is mediated by Fc receptor CD16 ($Fc\gamma RIII$ in mice, $Fc\gamma RIIIa/b$ in humans) which is expressed by NK cells. CD16 binds the Fc part of Abs and this cross-linking leads to antibody dependent cell cytotoxicity (ADCC) of target cells.

ILCs have been identified to initially arise from the fetal liver [53,54] but in adults they develop in the bone marrow from the same progenitor as B and T cells that is called CLP. The transcription factor Id2 (inhibitor of DNA binding2) is the main transcription factor responsible for the fate of cells to become an ILCs and no B or T cell. ILCs leave the bone marrow to populate lymphoid as well as peripheral organs (mucosal tissues), such as the dermis, liver, small intestine and lung, where they reside in the tissues and maintain homeostasis [24,52]. ILCs share transcription factors with T cells and produce similar cytokines. Therefore, ILCs have been divided into cytotoxic cells (NK cells), ILC1, ILC2 and ILC3 – mirroring the functions of T cell subsets: type 1, type 2 or type 17 T subpopulations [55].

ILC1s and NK cells are the closest related subsets within the ILC group and both cell type respond to IL-12 and IL-18 with the production of IFN-γ. However, they can be distinguished by their transcription factors which is the best way of discriminating these cell types: They are both $ROR\gamma t^{-}$ $T\text{-}Bet^{+}$ $GATA3^{int}$ but only NK cells are also $Eomes^{+}$ [55]. Other differences between

ILC1s and NK cells are that NK cells can be found in the circulation, while ILC1s seem to mainly be tissue-resident cells and NK cells are more similar to cytotoxic $CD8^+$ T cells whereas ILC1s seem to be more like $T_h1$ $CD4^+$ T cells.

## 1.3 MYELOID CELLS

Myeloid progenitors can develop in the bone marrow and give rise to granulocytes (basophils, eosinophils and neutrophils) and monocytes which can subsequently differentiate into macrophages or DCs. As the important myeloid cells for this thesis are the macrophages, only they will be described here in detail besides myeloid-derived suppressor cells (MDSCs).

### 1.3.1 Monocytes

Blood monocytes can be distinguished into two subsets based on their receptor expression and migratory capacities: In mice, there are $CX3CR1^{int}Ly6C^+$ (in humans $CD14^+CD16^-$) and $CX3CR1^{high}Ly6C^-$ cells (in humans $CD14^{+/mid}$ $CD16^+$). $CCR2^+CX3CR1^{int}Ly6C^+$ is the subset that is migrating towards the site of an inflammation whereas the $CCR2^{low}CX3CR1^{high}Ly6C^-$ subset is the "patrolling" monocyte [56,57]. In the absence of an inflammation, $CX3CR1^{int}Ly6C^+$ monocytes can travel back to the bone marrow and convert to $CX3CR1^{high}Ly6C^-$ [58]. Leaving the bone marrow and the recruitment to sites of inflammation is dependent on CCR2-CCL2 signaling [59].

### 1.3.2 Macrophages

Macrophages are professional phagocytes that belong to the innate immune system and are crucial for the clearance of pathogens but also involved in the maintenance of tissue homeostasis. Phagocytes were discovered by Ilya Metchnikoff in 1866 when he found that leukocytes were able to migrate to the site of infection and ingest pathogens. For this, he used starfish larvae and pricked them with thorns to activate their immune cells to attack the foreign invader. He named this process phagocytosis and the cells phagocytes from Greek *phago* = to devour and *cytos* = cells [1].

Macrophages are tissue-resident cells that are needed for clearing pathogens, removing old or damaged erythrocytes in the spleen and liver but also to phagocyte cells debris and apoptotic cells [60]. Besides that, they fulfill a plethora of other functions as the regulation of tissue homeostasis, tissue remodeling and initiating adaptive immune responses through cytokine and chemokine production [61]. The main regulator of macrophages is colony-stimulating factor 1 receptor (CSF1R) which ligand is CSF1. Macrophages rely on macrophage-colony stimulating factor (M-CSF) for their survival. Their name originates from Greek and means "large eaters", *makros* = large and *phagein* = eat.

#### *1.3.2.1 Ontogeny of macrophages*

For decades, it was believed that all macrophages origin from blood-circulating monocytes that derive from a BM progenitor and that these monocytes constantly replenish the pool of tissue-resident macrophages. This founded on the concept of the "mononuclear phagocyte system" proposed by van Furth in the 1970s [62]. However, the "mononuclear phagocyte system" has been refuted and fate-mapping studies revealed that most of the tissue-resident macrophages develop already during embryogenesis in the yolk sac or fetal liver and renew themselves during adulthood, such as microglia (brain), Kupffer cells (liver), alveolar (lung) and splenic red pulp (spleen) macrophages [63]. However, under inflammatory conditions circulating monocytes can still give rise to tissue-resident macrophages [64–66]. Moreover, a pool of monocytes resides in the spleen which can replenish tissue-resident macrophages and deals as reservoir of inflammatory monocytes that can be rapidly recruited in case of an inflammation [67]. In contrast, dermal macrophages and the ones found in the intestine are constantly replaced by blood circulating monocytes [68]. Taken together, most macrophage populations are maintaining themselves by local proliferation throughout the lifetime [68].

#### *1.3.2.2 Macrophage subsets*

Macrophages exist nearly in all tissues and can be found in lymphoid organs as well as in non-lymphoid tissues. They are very plastic cells which are affected by tissue-derived factors as part of their local education. Alveolar macrophages with fetal origin usually have a non-inflammatory state and equipped with many PRRs they are responsible for the removal of air-borne pathogens and apoptotic cells. Kupffer cells, the liver-resident macrophages, filter the blood not only for pathogens but also remove potentially harmful agents such as malformed erythrocytes, extracellular matrix components and regulate plasma cholesterol. Osteoclasts, the macrophages in the bones, are required for bone resorption and bone formation during embryogenesis. However, it is still disputed whether they origin pre- or postnatally. Microglia are the tissue-resident macrophages of the central nervous system and the first macrophages shown to be independent from bone marrow-derived monocytes. They exclusively derive from the embryonic yolk sac and replenish themselves during life. Macrophages are also present in the peritoneal cavity that rely on the transcription factor Gata-6 in contrast to other tissue-resident macrophages. Gata-6 is induced by retinoic acid that can be found in high amounts in the omentum and thus contributing to the tissue-specific education of these peritoneal macrophages [69]. As they are easy to access, peritoneal macrophages have been the subject of many studies about signaling, cytokine and chemokine production after pathogen stimulation and processes of phagocytosis. It is still largely unknown whether these cells origin from embryogenic progenitors or HSC in the bone marrow – however, there are two peritoneal macrophage populations F4/80$^{high}$ CD11b$^{high}$ (also referred to as large macrophages) and F4/80$^{int}$ CD11b$^{int}$ (also referred to as small macrophages) which might explain the origin. It has also been proposed that F4/80$^{int}$ CD11b$^{int}$ macrophages are a precursor for the mature F4/80$^{high}$ CD11b$^{high}$ macrophages [70 63 59]. Macrophages in the gut are sentinels and besides the removal of pathogens they maintain tolerance to the gut flora and food.

The spleen is the largest lymphoid organ and the main site where adaptive immunity to blood-born invaders is initiated. The spleen acts as a filter, is highly structured and homes several macrophage subsets with specific functions: Red pulp macrophages are the most prominent macrophages in the spleen, specialized in uptake of senescent or damaged red blood cells and recycling of heme and iron [71,72]. The red pulp is also the place where monocytes are stored that can react rapidly in case of inflammation and migrate to the site of inflammation [67]. The splenic white pulp contains macrophages within the GC regions called tingible- body macrophages removing apoptotic B cells during GC reactions [59]. The boundary between red and white pulp is the MZ and contains two highly specialized macrophage subtypes: marginal zone macrophages (MZMs) and metallophilic macrophages (MMs). When blood enters the spleen, the blood flow slows down and macrophages screen for potential threats and remove apoptotic cells. Moreover, MZMs are responsible to inhibit immune responses against apoptotic cells [73]. MMs align the inner layer of the MZ whereas MZMs are located on the outer site of the MZ. Both macrophage subsets are dependent on the transcription of nuclear liver-X receptor α [74]. MMs are characterized by the expression of CD169 (Siglec-1, sialoadhesin) which allows them to take up sialic acid-expressing bacteria [75].

MZMs are of particular interest for this thesis as they express the class A scavenger receptor MARCO (macrophage receptor with collagenous structure) [44,76–78] that is described in more detail later. MZMs are scaffolded with many PRRs for the uptake and clearance of blood-borne pathogens. Besides MARCO they express C-type lectin CD209b (SIGN-RI). Upon the activation of MZMs with LPS the receptors behave differently: Whereas SIGN-RI is downregulated, MARCO engagement leads to the release of pro-inflammatory cytokines [79–81]. The expression of MARCO on MZMs is important for the retention of MZBs [10] and moreover, the colocalization of MZMs and MZBs is required for early IgM production by MZBs in response to bacteria. This strongly suggests that MZMs can present Ags to MZBs to support early responses against polysaccharides [82]. In turn, MZBs release chemokines, such as CCL19 and CCL21, that are needed for the retention of MZMs [83] and regulate their SIGN-RI expression [38]. Binding of intravenous Ig (IVIG) to SIGN-RI on MZMs leads to the mediation of anti-inflammatory responses due to the engagement of inhibitory FcγRIIb. IVIG is commonly used for treating chronic autoimmune diseases because it inhibits autoantibody-mediated inflammation by increasing the activation threshold [84,85].

Lymph nodes show a similar structure compared to the spleen and filter the lymph for lymph-borne pathogens. There are three distinct macrophage subsets present in the lymph nodes: medullary sinus macrophages (MSMs), medullary cord macrophages (MCMs) and subcapsular sinus macrophages (SSMs) [86]. Medullary macrophages also express MARCO to efficiently clear pathogens and foreign particles from the lymph [78]. In steady state, the medulla represents a small part of the lymph node but can become enlarged upon plasma cell response [86]. The SSMs are $CD169^{+}$ such as MMs in the spleen. SSMs can ingest viruses from the lymph circulation and initiate antiviral humoral immune responses by transferring the Ag to B cells [87]. MSMs also express CD169 [86].

### *1.3.2.3 Macrophage polarization*

Macrophages are very plastic cells that can change phenotypically but also on transcriptional level dependent on their microenvironment and the surrounding stimuli. The first time the term "macrophage activation" was introduced was in 1962 by Mackaness. He recorded enhanced microbicidal capacity of macrophages against several microorganisms [88] which two decades later traced back to be dependent on IFN-γ and that the activation of macrophages also changes their metabolism [89]. In 1992, Stein et al. discovered the alternative activation of macrophages that was independent on IFN-γ but dependent on IL-4 stimulation leading to different phenotypic changes than the classical activation including the expression of mannose receptor (CD206) [90]. These were the beginnings of the attempt to classify the complex and diverse macrophage populations. Mills et al. were the first to define the M1/M2 axis for macrophage polarization according to their function and to mimic the $T_h1$ and $T_h2$ T cell subsets. He defined "M1 to be able to kill whereas M2 macrophages are able to heal" [91]. Thus, macrophages have been distinguished into two extreme states and the M1 term was further used for classically activated macrophages [89] and M2 as alternatively activated macrophage [92,93]. Later based on the great plasticity of macrophages, Mosser and Edwards proposed a classification with continuous transitions instead of two extremes (M1 and M2) in form of a colored wheel and suggested three main macrophage functions: immune regulation, wound healing and host defense, but with transition states in between these main subtypes [94].

The classical activated M1 macrophages are pro-inflammatory and involved in host defense. They arise upon stimulation with IFN-γ and/or TLR ligands, such as LPS. IFN- γ signals through STAT1 and interferon regulatory factors (IRF), such as IRF-1 and IRF-8, inducing cytokine receptors, such as IL-15R , IL-2R and IL-6R [93]. IFN-γ can be produced by many cell types and one important source are activated NK cells which then support the shift to M1 macrophages, expression of pro-inflammatory cytokines and enhance their killing capacity [95,96]. LPS activates MyD88-dependent signaling pathways resulting in the expression of pro-inflammatory cytokines, such as IL-12, IL-6, IL-1β , TNF, IL-23; chemokines, such as CXCL10 , CXCL11 and CCL2; co-stimulatory molecules, such as CD86; and MHC-II molecules for increased Ag presentation [93,94]. IL-23 has been shown to be crucial for the expansion of $T_h17$ cells which in turn secrete TNF-α and IL-6 supporting an inflammatory milieu [97]. Recently, granulocyte-macrophage colony-stimulating factor (GM-CSF) has also been defined as stimulator for M1 macrophages [98]. Furthermore, M1 macrophages have increased expression of *Nos2* which is encoding for the enzyme inducible nitric oxide synthase (iNOS). iNOS metabolizes L-arginine and synthesizes nitric oxide (NO) [99]. Thus, increased NO production results in augmented production of reactive oxygen species (ROS) and therefore to enhanced cytotoxic function against pathogens [100]. Therefore, pro-inflammatory M1 macrophages are important players in host defense and competent in clearing pathogens. However, M1 macrophages responses can also damage host tissues and they are known key players in many autoimmune diseases, such as rheumatoid arthritis [101,102].

In contrast, alternatively activated M2 macrophages can be attained by a broader mix of stimuli which is why they are subdivided further [93,103,104] into M2a, M2b, M2c and sometimes also M2d macrophages (Figure 2). M2a macrophages get activated upon stimulation with IL-4 and/or IL-13 and are IL4Rα-dependent. IL-4 is secreted by granulocytes, such as basophils and mast cells, in response to injury or the occurrence of parasites or fungi and signals through STAT6. It further induces Arginase-1 expression which converts L-arginine to ornithine and urea, precursors of polyamines and collagen, and thus supporting the production of

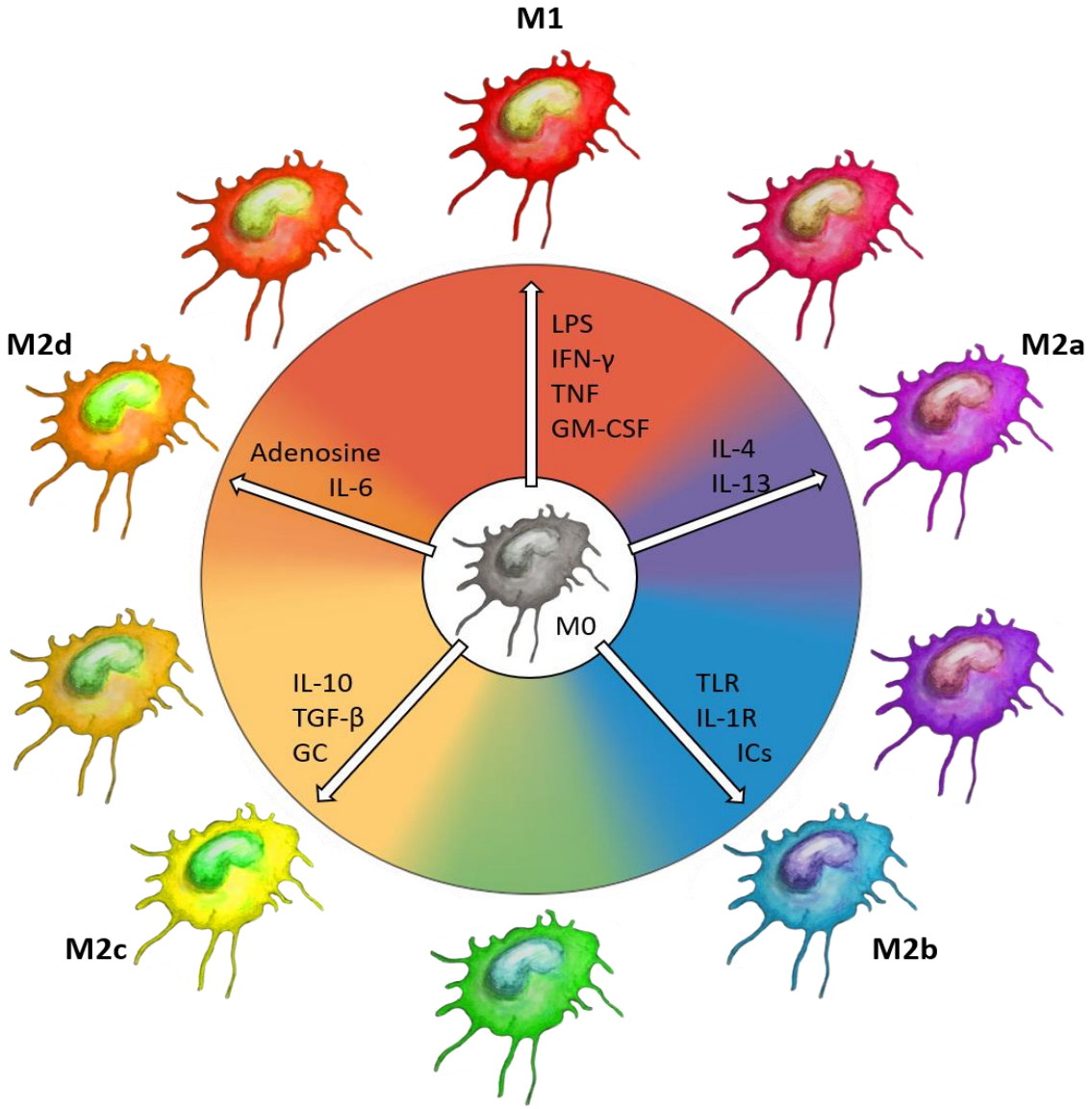


**Figure 2: Diversity of macrophage activation represented by a colored wheel.** Macrophages can quickly change between activation states depending on the factors they are exposed to which is illustrated here by a colored wheel with flowing transitions. The cells "between" the defined states represent macrophages that share properties or are in a transitional phase and are therefore difficult to classify. The main pro-inflammatory state is shown by M1 macrophages, normally induced by $T_H1$ responses or infection and cytokines such as TNF and IFN. M2 macrophages are more diverse with anti-inflammatory, immune suppressive and pro-tumoral properties and have been subdivided further: M2a are similar to wound-healing macrophages and need IL-4/IL-13. M2b and M2c are similar to regulatory M2-like macrophages and induced by TLR or IL-1R ligands and immune complexes; IL-10, TGF-β and glucocorticoids, respectively. M2d macrophages arise upon adenosine or IL-6 stimulation. TAMs are a heterogenous population and share characteristics of different activation states dependent on the TME, demonstrated here by the colors between other activation states. Adapted from reference #94.

extracellular matrix components [105] and upregulates mannose receptor CD206 [90]. Also, IL-4 and IL-13 stimulated macrophages show decreased killing capacity compared to M1 macrophages [94]. M2b macrophages get activated in response to immune complexes and TLR ligands or IL-1R ligands. The recognition of immune complexes by Fc receptors recruits tyrosine kinase Spleen tyrosine kinase (Syk) and activates phosphoinositide 3-kinase (PI3K). It has also been shown that ligation of FcγRs on macrophages using Abs increased IL-10 production in response to LPS [106,107]. M2c macrophages are activated by IL-10, glucocorticoids and TGF-β. Glucocorticoids are known immune suppressive agents [108] which diffuse through the membrane of macrophages binding the glucocorticoid receptor- α and thus activating IL-10 production [109]. M2d activation appears after exposure to IL-6 and adenosine [103,110,111] leading to an angiogenic switch including vascular endothelial growth factor (VEGF) expression in an IL-4Rα-independent way [112,113]. Recently, it has also been shown in human monocytes that M-CSF gives rise to M2-like cells with high IL-10 production [98].

M2 macrophages express immune-suppressive and -modulatory cytokines, such as IL-10 and high levels of Arginase-1 (Arg-1) that depletes arginine from the surrounding and thus inhibiting T cell proliferation. IL-10 has been shown to enhance levels of IL-4R on the cell surface and thus making macrophages more susceptible to IL-4 and IL-13 signaling resulting in M2a polarization in a STAT3-dependent way [114]. Furthermore, chitinases and chitinase-like molecules, YM1 (also known as *CHI3L3*) and YM2, resistin-like molecule-alpha (RELMa, also known as *FIZZ1*) are upregulated. Through the expression of these molecules these macrophages are suggested to be involved in the degradation of chitin-containing surfaces of parasites and fungi and that they play a role in matrix reorganization and wound healing by their matrix-binding ability [94]. This type of macrophages has been connected to experimental asthma in mice and to be the mediators of tissue fibrosis in chronic schistosomiasis due to their malfunctioning regulation of matrix-enhancing possibilities [94]. Tumor-associated macrophages (TAMs) are also considered as M2-like macrophages as they are immune suppressive and pro-tumorigenic but are difficult to classify in any of the macrophage subpopulations as they differ between tumors.

Taken together, the classification of macrophages into subgroups dependent on their functions or cytokines they respond to is difficult due to their complexity and diversity, especially *in vivo*. Also in humans, macrophages are very plastic cells and transcriptome analysis of differently stimulated human peripheral blood monocyte cell (PBMC)-derived macrophages revealed a plethora of macrophage subpopulations [115]. Macrophages need to be discussed in the context they are studied in. Regardless of which phenotype the cells have when entering a new tissue or situation, they will adapt and change their polarization quickly to what is required there. Further effort needs to be put into exploring and understanding the whole capacity of macrophages.

#### *1.3.2.4 Metabolic macrophage polarization*

Metabolic processes are complex, and the numbers of metabolites can easily run into the thousands. The last decades, metabolism got more attention in the field of immunology as it has been demonstrated that immune cells not only change their metabolic program in response to nutrients and oxygen availability but also upon activation and engagement of PRRs. In the 1960s, phagocytosis in leukocytes has been connected to increased oxygen consumption and glycolysis whereas alveolar macrophages were described to use oxidative phosphorylation when phagocytosing [116,117]. In 1970, Hard investigated metabolic processes specifically in macrophages and found that activated peritoneal macrophages show higher levels of glycolysis and lower levels of oxygen consumption [118]. Stimulation of macrophages with LPS shifts their metabolic program to increased glycolysis leading to a pro-inflammatory phenotype including IL-1β secretion [119,120] and also IFN-γ stimulation changes their metabolism [89]. In 2000, Mills et al. discovered important differences in the metabolism of M1 and M2 macrophages: M1 macrophages consume arginine for the production of NO required for pathogen killing; M2 macrophages use arginine to produce urea, polyamines and ornithine that are needed for repair and wound healing processes, such as collagen biosynthesis [91 121]. Therefore, the polarization state of macrophages cannot only be found in gene expression and surface markers but also in their metabolic programming; which is why the field of immunometabolism got tremendous interest in the recent years.

The shift towards enhanced glycolysis in LPS-activated macrophages is called "Warburg effect". Otto Warburg detected increased glycolytic activity in tumor cells under aerobic conditions even though mitochondrial oxidative phosphorylation is more efficient regarding ATP production. However, instead of entering the tricarboxylic acid cycle and subsequent oxidative phosphorylation (OXPHOS), pyruvate was converted to lactate because lactic acid fermentation generates additional metabolites to support proliferation [122]. Furthermore, lactate acid as well as acidic pH has been shown to polarize macrophages towards the tumor-supporting M2 macrophage with increased VEGFA and Arginase-1 expression [123,124]. In activated pro-inflammatory M1 macrophages the same shift from OXPHOS towards glycolysis has been observed, similar to the Warburg Effect. This has also been demonstrated in other immune cells, such as neutrophils as they rely on aerobic glycolysis for ATP production and only consume oxygen for the production of hydrogen peroxide ($H_2O_2$) needed for killing [125,126]. Classically activated M1 macrophages usually need rapid energy sources for efficient pro-inflammatory responses. Therefore, they upregulate glycolytic processes to quickly gain energy in form of ATP. M1 macrophages have been shown to have a "broken" Krebs cycle which leads to the accumulation of Krebs cycle intermediates citrate and succinate [127]. Citrate is further important for the production of NO, ROS and prostaglandins as inflammatory mediators [119]. In contrast, the accumulation of succinate induces IL-1β secretion. High succinate concentrations activate hypoxia-inducible factor 1-alpha (HIF-1α) that directly induces pro-IL-1β expression by binding to the IL-1β promoter. The NACHT, LRR and PYD domains-containing protein 3 (NALP3) inflammasome, encoded by the NLRP3 gene, can also be activated through enhanced glycolysis [120,128]. Also in human M1 macrophages increased

glycolysis correlates with an inflammatory phenotype [129]. Taken together, all these processes supply cells with rapid energy and products they need to exert their inflammatory activity. In contrast, alternatively activated M2 macrophages rely on mitochondrial respiration and obtain their energy from fatty acid oxidation and oxidative metabolism [121].

Immunometabolism is also approached in other immune cells, such as T cells and NK cells. Activated T cells undergo metabolic reprogramming and increase aerobic glycolysis [130,131] and also NK cells enhance glycolysis levels and OXPHOS after activation with IL-12 and IL-15 *in vitro,* in mice as well as in humans. However, chronic exposure of human NK cells to IL-15 *in vitro* decreased their metabolic processes [132].

### 1.3.3 Myeloid- derived suppressor cells

Myeloid- derived suppressor cells (MDSCs) are cells with immunosuppressive potential that can strongly inhibit T cell responses but also innate immune responses by affecting cytokine production by macrophages. MDSCs are no defined population of cells but a phenotypically heterogeneous group that consists of immature myeloid cells (IMC). IMCs occur during normal myelopoiesis in the bone marrow but usually migrate to the periphery and differentiate into macrophages, DCs or granulocytes. However, under certain pathological circumstances the differentiation of IMCs is blocked, and these cells are expanding and are then called MDSCs. They express a plethora of immune inhibiting factors, such as Arginase-1, iNOS and enhance production of ROS and NO. There are two main MDSCs subtypes that are distinct in their surface receptor expression: In mice, granulocytic MDSCs ($CD11b^+$ $Ly6G^+$ $Ly6C^{low}$) and monocytic MDSCs ($CD11b^+$ $Ly6G^-$ $Ly6C^{high}$); in humans, $CD11b^+CD14^-$ $CD33^+$ or $LIN^-$ $HLA^-DR^-$ $CD33^+$ [133]. MDSCs in the pathological condition of cancer is further described in chapter 1.5.

## 1.4 SCAVENGER RECEPTORS

Initially, scavenger receptors (SR) have been defined by their ability to bind and internalize modified low-density lipoprotein in 1979 by Goldstein and colleagues [134]. Since then, many other ligands have been defined and SR are known to bind polyanionic (negatively charged) molecules and contain highly conserved scavenger receptor cysteine-rich (SRCR) domains [135]. SR are PRRs on phagocytes that can bind modified self, such as acetylated or oxidized low density lipoprotein (LDL) [134] and bacterial structures, such as LPS by gram-negative bacteria [136] and lipoteichoic acid by gram-positive bacteria. SR are classified into eight different groups based on their multi- domain structure (class A – H) [135,137]; however, the structures between the groups are very heterogeneous.

Based on their binding to LPS, SR have been suggested to be involved in anti-bacterial responses and to play a crucial role in the host defense against pathogens. They can bind and ingest bacteria directly such as *Escherichia Coli* [138] and SR-deficient mice implicated to be more susceptible to infection with *Listeria monocytogenes, herpes simplex virus type-1* [139] or *Staphylococcus aureus* [140]. Members of the scavenger receptor class-A (SR-A) family are trimeric transmembrane glycoproteins [141]. Several SRs are involved in the clearance of

apoptotic cells, such as SR-A family members and CD36 [142], and it has been shown that SR-A are crucial for the internalization of extracellular double stranded RNA [143,144]. Furthermore, SR-A receptors have been revealed to bind ligands through their collagenous domain of the receptor [145,146] and their short cytoplasmic domain to be crucial for trafficking and internalization [147,148]. As scavenger receptor MARCO is the central topic of this thesis it will be described in detail below.

### 1.4.1 Scavenger receptor MARCO

Scavenger receptor MARCO (macrophage receptor with collagenous structure) was cloned by Karl Tryggvason at Karolinska Institutet in 1995. It is a 210 kDa trimeric membrane bound type II glycoprotein with collagenous structure belonging to the SR-A family. The receptor consists of five regions (Figure 3): a N- terminal short cytoplasmic domain, a transmembrane domain, a spacer domain between collagenous structure and membrane, the collagenous structure that is involved in trimerization and the C-terminal group A-type SRCR region [77,149] which has been implicated in ligand binding [150]. MARCO expression is restricted to some macrophage subtypes and is constitutively expressed on MZMs in the spleen, peritoneal macrophages, medullary macrophages in the lymph node [149] and on alveolar macrophages [151].

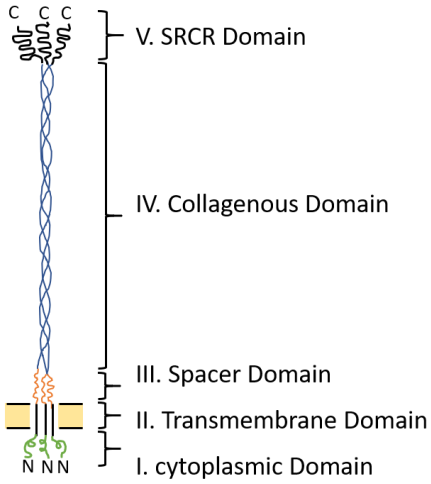


**Figure 3: The structure of scavenger receptor MARCO**. Schematic representation of its five domains.

In humans, highest MARCO expression is found in lymph nodes, liver and on alveolar macrophages [152,153]. Even though it is not expressed on other macrophage subtypes in steady state, MARCO can be upregulated upon treatment with bacteria or LPS stimulation on liver-resident Kupffer cells [154], DCs [155], red pulp macrophages [154] and other macrophages [156]. LPS-induced MARCO upregulation in splenic macrophages is Toll like receptor 4 (TLR4)-dependent and can occur in a MyD88-dependent or MyD88-independent manner [157]. Also SR-A has been reported to be upregulated on murine macrophages upon LPS stimulation [158] and even though SR-A and MARCO have structural similarities, they act differently on the macrophage: Ligation of SR-A inhibits IL-12 production, whereas engagement of MARCO supports IL-12 release [81,159].

In lymphoid organs, MARCO expressing cells are strategically well-located aligning the marginal zone in the spleen and the medullary cord of lymph nodes which highlights MARCO as a crucial receptor for clearance of blood- and lymph-borne pathogens and apoptotic cells [77]. Defects in clearing apoptotic cells bears a higher risk to develop autoimmune diseases, such as SLE [160]. Besides the disease-characteristic presence of anti-DNA Abs, SLE patients also have autoantibodies against MARCO [44] and when injecting apoptotic cells into MARCO-deficient mice they develop higher levels of anti-DNA Abs [44]. However, it is not known whether these autoantibodies are initiators or symptoms of the disease. Other known ligands for MARCO are

oxidized LDL [134], *E.Coli* and *S. aureus* [78]. MARCO can deliver CpG DNA to the endosomal TLR9 and is required for TLR9–mediated IL-12 and NO production in response to CpG oligonucleotides in microbial DNA [161]. Lacking MARCO expression on splenic MZMs causes the disruption of functional MZ structure and impairs responses to TI Ag [162]. The lack of MARCO also reduces the number of peritoneal macrophages in MARCO-deficient mice [162]. When non-myeloid cells are transfected to express MARCO they change their cell morphology obtaining large lamellipodia-like structures and dendritic-like membrane extensions [163]. Also the transfection of macrophages with SR-A results in morphological changes including increased spreading [164]. This results in decreased migration which is important for successful particle engulfment [163].

So far, little is known about signal transduction after engagement of MARCO. The receptor has a very short cytoplasmic domain and does not contain any classical sequences that are associated with internalization, phosphorylation or clustering. In microglia, MARCO has been reported to interact with formyl peptide receptors which are G-protein-coupled PRRs that recognize a broad variety of ligands, derived from pathogens but also host cells [165]. Further, MARCO mediates inflammatory cytokine production upon *Mycobacterium tuberculosis* infection through TLR2 and CD14 [166] and enhances TLR2- and nucleotide-binding oligomerization domain 2 (NOD2)-dependent signaling during *S. pneumoniae* colonization in the nasopharynx of mice. Thus, increasing NF- κB activation and enhancing pro-inflammatory molecule production, such as TNF-α, IL-1β, and IL-6 [167]. Additionally, ligand binding and internalization by MARCO increases cytosolic NOD2 and NALP3 inflammasome as well as endosomal TLR3 signaling. At the same time, MARCO ligand internalization diminishes TLR4-dependent responses [168]. When targeting MARCO with monoclonal Abs, $MARCO^{+}$ peritoneal macrophages release high levels of ATP comparable to the levels after LPS stimulation which we demonstrate in this thesis in paper I [169]. Extracellular ATP is seen as pro-inflammatory agent and leads to M1 macrophage polarization [170]. In contrast, MARCO inhibits early inflammatory responses to *influenza A virus* and MARCO-deficient mice show increased neutrophil influx and better survival [171]. The anti-MARCO Ab clone used in the studies included in this thesis has been demonstrated to block ligand binding as it suppressed the uptake of FITC-labelled *E.coli* by MARCO expressing/transfected CHO cells [156]. This suggests that binding of anti-MARCO Ab to MARCO could initiate Ab uptake in the same way as it initiates the uptake of MARCO after bacteria binding. By using cells expressing truncated versions of MARCO and measuring the uptake of FITC-labeled *E.coli* the bacteria binding site was found to be located immediately proximal to the cysteine residues of the SRCR domain [149]. This is supported by Novakowski et al. also indicating that the SRCR domain of MARCO is important for ligand binding unlike SR-A that binds its ligands through its collagenous structure [145,146]. Also, the SRCR domain of MARCO is required for the induction of pro-inflammatory signaling via TLR2 [172] and increasing TLR2 and CD14 signaling in response to *S. pneumoniae* [167].

Interestingly, MyD88-dependent TLR ligation and incubation with tumor lysate induces MARCO expression on DCs [173–175] which in turn enhances their phagocytic capacity [173].

However, another study by Granucci and colleagues shows that MARCO expression in DCs leads to actin cytoskeleton rearrangement similar to mature DC phenotype and a decrease in phagocytosis [155]. Targeting tumor lysate-conditioned MARCO$^+$ DCs with monoclonal Abs results in enhanced motility and anti-tumor activity in the B16 melanoma mouse model. Moreover, these anti-MARCO Ab targeted DCs lose their dendritic- like phenotype [174,176]. The follow up study in MARCO-deficient tumor-pulsed DCs showed increased migratory capacities toward the lymph nodes and enhanced anti-tumor T cell responses after injection into tumor-bearing mice [176]. This is supported by another study demonstrating that MARCO-deficient pulmonary DCs acquired increased migratory capacities towards the lymph nodes upon allergen exposure leading to augmented airway inflammation [177]. In contrast, alveolar macrophages require MARCO expression for effective lung defense [178].

In humans, MARCO (hMARCO) has been identified to be similar to murine MARCO in 68% amino acid sequence identity and 77% similarity [149]. In line with murine MARCO, hMARCO also binds *E.coli* and *S.aureus*; however, it was not found to bind modified LDL as its murine counterpart. Highest hMARCO expression in the human body was found in liver, lymph nodes and lung [152]. In patients that died from sepsis, widespread MARCO expression was found in several human tissues which supports previous findings in mice that MARCO can be upregulated upon bacterial infections [149,156]. MARCO has also been found on the immune suppressive TAM subtype in human non-small cell lung cancer (NSCLC) [179]. Within studies including in this thesis, we also found MARCO expression in other human cancers correlating with poor prognosis, including human melanoma, breast cancer and pancreatic cancer patients (paper II and IV). Interestingly, a recent study by Sun and colleagues reports a connection between MARCO and hepatocellular carcinoma (HCC) patients as they find MARCO expression correlates positively with patient survival rates. Fewer MARCO$^+$ macrophages were inside the tumor compared to peritumoral areas and MARCO expression decreases with tumor progression – correlating highest MARCO expression in healthy controls and lowest expression in HCC patients with metastasis [180].

## 1.5 THE TUMOR MICROENVIRONMENT

Tumors are complex and heterogeneous tissues that consist of many different components besides malignant cancer cells. The hallmarks of cancer progression and their characteristics were defined by Hanahan and Weinberg and include high replicative potential, genome instability, persisting growth but resisting apoptosis signals, abnormal metabolic processes, avoiding immune destruction, persistent angiogenesis, invasion and metastasis [181]. However, tumor cells do not operate alone in the complex multi-level process of tumor initiation, progression and metastasis spreading. Besides malignant cells there are many non-malignant cells involved, such as pericytes, adipocytes, cancer-associated fibroblasts (CAFs), endothelial cells and immune cells. In many solid tumors desmoplasia occurs, resulting in dense fibrosis and pancreatic cancer is one of the most extreme tumor types with fibrotic stroma.

The interaction between tumor and non-malignant cells defines the tumor microenvironment (TME) and consists of a complicated network between growth factors, cytokines, chemokines

but also inflammatory and matrix remodeling molecules [182]. Tumor cells organize their own microenvironment and tumor-derived mediators recruit other cell types to support their growth [182]. In the last decades, tumor-promoting inflammation and the escape from immune control became a hallmark of cancer [181] after Rudolf Virchow proposed a link between cancer and inflammation already in 1863. Immune cells are known to infiltrate most types of cancers; T and B lymphocytes, NK and NKT cells, MDSCs, tumor-associated neutrophils (TAN), DCs and TAMs are present. Most of these cells show an immunosuppressive phenotype that supports tumor growth and inhibits efficient anti-cancer immune responses (Figure 4). Especially TAMs play an important role in tumor-promoting inflammation and have been associated with poor prognosis in many human cancers [183].

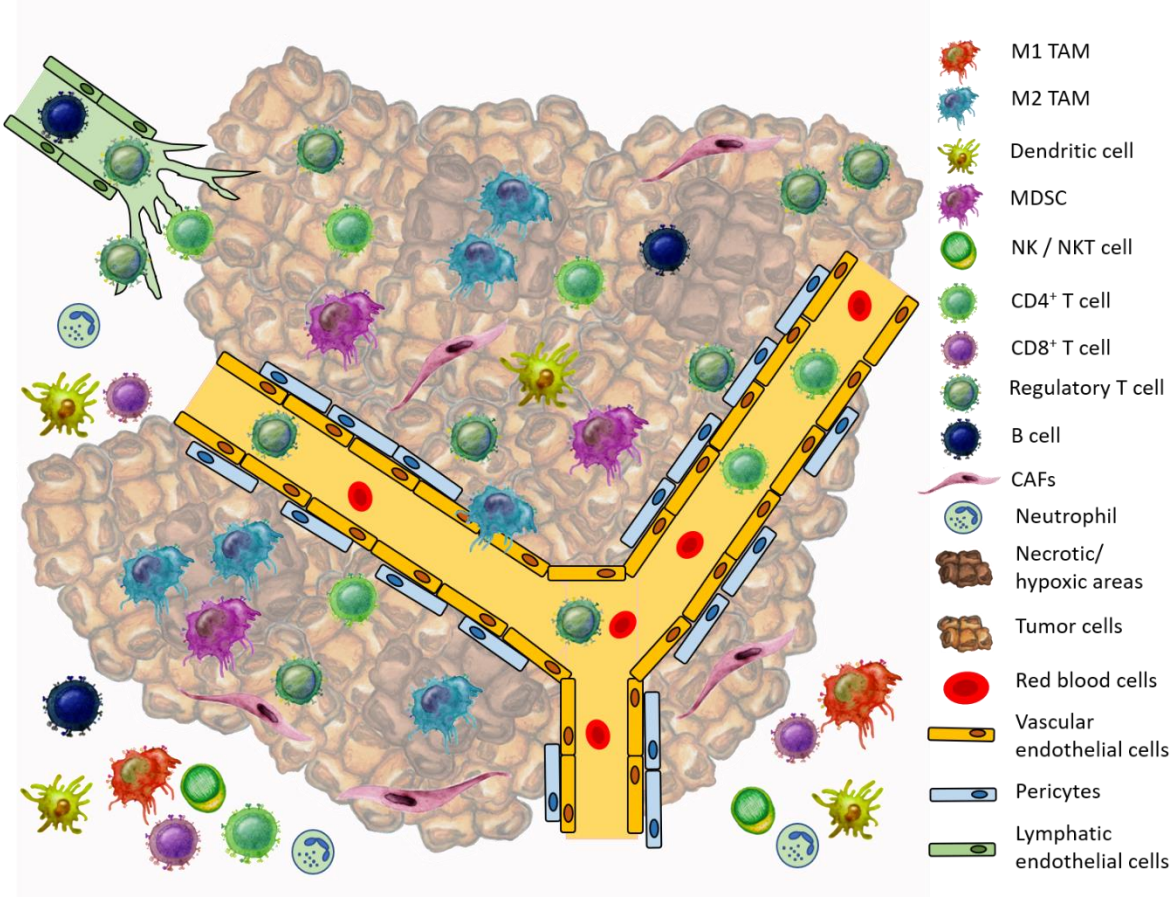


**Figure 4: Illustration of the immune suppressive tumor microenvironment.** The tumor microenvironment (TME) is very diverse and consists of many different cell types. Immune cells are recruited to the tumor site, re-programmed and suppressed by tumor-derived mediators. B cells, T cells, tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSC), neutrophils and dendritic cells are present. Besides immune cells, there are blood vessels, lymphatic vessels, pericytes, cancer-associated fibroblasts (CAFs) in the TME as well as hypoxic and necrotic areas, all supporting the tumor and its immune suppressive environment in their own ways.

The regulation of tumor growth and modulation of TME by the immune system has been a central question in the field of immunology already for years. It started with Burnet and Thomas when they proposed the "cancer immunosurveillance hypothesis" stating that immune cells were required for hampering cancer progression [184,185]. With the rise of improved mouse models of immunodeficiencies, immunologists performed additional experiments and further defined the involvement of immune cells in shaping the immunogenicity of tumors. Interestingly, immunocompetent mice had lesser immunogenic tumors ("edited") than mice

lacking an intact immune system ("unedited"). This uncovered the double-sided role of immune cells in tumor-fighting and tumor-promoting actions [186] and gave rise to the "cancer immunoediting hypothesis". Today we know that the immune system cannot only combat and fight tumors but also supports tumor growth on different levels in dynamic processes [187,188]. The immunoediting process of tumor cells can broadly be divided into three distinct phases: elimination, equilibrium and escape. However, malignant cells may skip phases dependent on their external influences. In the first step, the "elimination" phase, innate and adaptive immune cells collaboratively destroy and eliminate tumor cells. This can be due to the recognition of DAMPs from dying tumor or host cells or the activation of DCs through type I interferons and subsequent activation of adaptive immunity. The "equilibrium" stage is entered by tumor cells that survived the elimination phase. Here, tumor cell growth is controlled by the immune system and tumor cells are in a state of dormancy. This phase`s duration is very variable and can take up to years before tumor cells pass over to the escape phase. Once the tumor cells overcome equilibrium, they are entering the final "escape" phase. By that time, tumor cells acquired the capacity to evade the immune system and are "invisible" to the immune cells resulting in tumor cell outgrowth. This can be reached through the decrease or loss of tumor antigens or enhanced resistance against cytotoxic mechanisms. Furthermore, tumor cells can modulate the TME to become immune suppressive and thus, promoting their own survival and growth[187,188]. For instance, one way to escape the destruction by phagocytosis is the transmembrane protein CD47 which acts as "don`t eat me signal". CD47 binds its ligand signal regulatory protein-alpha (SIRP-α) expressed by TAMs and DCs and thus inhibits phagocytosis of the tumor cell. High intratumorally expression of CD47 has been correlated with bad prognosis in several cancers, such as breast cancer, and blocking of CD47 results in enhanced tumor cell killing by TAMs [189].

Some of the immune suppressive properties immune cells can exert in the TME are listed here: $T_{regs}$ are defined by their expression of CD25 and transcription factor FoxP3, elicit immunosuppressive functions through the release of IL-10 and TGF-β and cytotoxic T-lymphocyte antigen 4 (CTLA-4) which is a negative regulator and inhibits further initiation of potent anti-tumor immune responses [190,191]. Therefore, the presence of $T_{regs}$ is associated with bad prognosis. Also tumor-infiltrating B cells have been found to be pro-tumorigenic as B cell depletion slowed down tumor growth and metastases formation to the lung [192]. The discovery of regulatory B cells producing IL-10 and TGF-β confirmed the immunosuppressive capacity of B cells inside the tumor and regulatory B cells help $CD4^+$ T cells to differentiate into tumor-promoting $T_{regs}$ [193]. CAFs can secrete many growth factors and cytokines, including TGF-β which supports epithelial-mesenchymal transition (EMT) in tumor cells and thus promoting metastases spreading to distal tissues [194,195]. In addition, vascularization is required for tumor growth and immune cells can release angiogenic factors supporting neovascularization within the tumor. VEGF is the prevailing pro-angiogenic factor in the TME that initiates new vessel formation characterized by abnormal structures, such as diffuse branching and irregular vessel lumen. Intratumor vascularization is often also leaky hence supporting the intravasation of tumor cells [196].

As described in chapter 1.3.3, MDSCs are defined as potent inhibitory immune cells within the TME in many cancers and it has been demonstrated that they can also differentiate into TAMs [197]. In healthy individuals, there are only few of these immature myeloid cells present. In mice, there are usually 2- 4% in the spleen; however, in tumor-bearing mice this highly suppressive cell type can expand and reaches 20-40% of all splenic cells. Factors that have been shown to result in MDSC expansion are prostaglandins, M-CSF, GM-CSF, IL-6 and VEGF – all factors that are present in most tumors. On the transcriptional level, STAT3 was identified to be the main regulator of MDSC expansion. To get activated, MDSCs need to experience IFN-γ, IL-4, IL-13 or TGF-β [133] and characteristically for MDSCs is the increased expression of Arginase-1 and iNOS. These enzymes consume L-arginine, thus inhibiting T cell proliferation [198] and the generation of NO reduces T cell activation by inducing apoptosis [133,199]. Further, MDSCs support $T_{reg}$ differentiation and promote the formation of an immune suppressive TME [133,200]. In relation to TAMs, it has been shown that the adoptive transfer of freshly isolated MDSCs to tumor – bearing mice results in differentiation of MDSCs to TAMs. These TAMs were able to induce T cell apoptosis *in vitro* in an Arginase-1 and NO-dependent matter [201].

All these immune cells contribute to the immune suppressive TME which inhibits anti-tumor responses and at the same time promotes tumor growth. In the next chapter, I will further focus on TAMs as the main player in this thesis, their role in the TME and some processes of particular interest for understanding the studies included in this thesis.

### 1.5.1 Tumor-associated macrophages

Macrophages in general are a very heterogenous cell population and difficult to classify. TAMs are even more complex and their presence is often correlated with poor prognosis [202,203]. They can be of pro-inflammatory nature inducing potent anti-tumor responses resulting in the eliminiation of tumor cells. However, TAMs are mostly of a M2-like immune suppressive phenotype with pro-tumor capacities supporting tumor growth by promoting angiogenesis, tumor cell proliferation, tissue remodeling and metastases.

#### *1.5.1.1 Origin of tumor-associated macrophages*

The origin of TAMs is still disputed and TAMs were shown to originate from tissue- resident macrophages, circulating bone marrow-derived monocytes but also from M-MDSC that are recruited to the tumor site by mainly CCL-2 [203,204] and CCL-5 [205]. These chemokines can be released by tumor cells, endothelial cells, fibroblasts but also TAMs [205]. Additionally, CCL3 (macrophage inflammatory protein (MIP)1 alpha), CCL4 (MIP1 beta), CXCL12 (stromal cell-derived factor 1 alpha), IL-6, CSF-1 and VEGF-α are capable of recruiting monocytes to the tumor site in different mouse models [206].

In mammary adenocarcinoma, $Ly6C^{hi}CX_3CR1^{low}$ monocytes have been identified as the main precursors of TAMs [207]. This is consistent with the findings of Franklin and colleagues demonstrating that circulating $Ly6C^{+}CCR2^{+}$ monocytes replenish TAMs in the MMTV-PyMT tumor model [208]. Also, the spleen has been identified as a reservoir of $Ly6C^{high}$ monocytes that can be recruited to tumors via CCL2/CCR2 signaling and further differentiate into TAMs [209].

However, TAMs can also originate from tissue-resident macrophages, such as in gliomas with yolk sac-derived progenitors [210] or even represent a mixed pool of tissue-resident and inflammatory monocyte-derived TAMs as reported by Zhu et al. In murine pancreatic ductal adenocarcinoma (PDAC) models, Zhu and colleagues identified TAMs originated from pancreas-resident macrophages and others from inflammatory monocytes. Interestingly, the tissue-resident macrophages arose from embryonic stem cells and proliferated during tumor progression. Comparing these two TAM subpopulations by mRNA sequencing, only the embryonic-derived tissue-resident TAMs showed an increased pro-fibrogenic phenotype suggesting a role in remodeling the extracellular matrix while the monocyte-derived TAMs showed enhanced gene expression related to Ag presentation [211]. Furthermore, MDSCs can quickly differentiate into immune suppressive TAMs when they infiltrate tumors. Hypoxia leads to dramatic changes in MDSCs via HIF-1α, associated with the upregulation of Arg-1 and iNOS and differentiation into immune suppressive TAMs [197]. A recent study proposed the differentiation of MDSCs to TAMs is caused by hypoxia – mediated decrease of STAT3. However, they found it to be independent on HIF-1α but downregulation of STAT3 resulted in differentiation of M-MDSCs to TAMs [212].

#### *1.5.1.2 The role of tumor-associated macrophages in the tumor*

TAMs are one of the most abundant immune cell in most cancers and exert numerous functions within the TME to affect tumor growth: On one hand they can inhibit tumor growth and eliminate malignant cells, but on the other hand they also support tumor progression by expression of immune suppressive cytokines, degradation of extracellular matrix, stimulation of angiogenesis [213] or even promote tumor cell intravasation to form distant metastases [214]. For this, tumor cells express CSF-1 to stimulate TAMs and in turn, macrophages release epidermal growth factor (EGF), a known tumor cell growth factor.

Mostly, TAMs have an activation state that is towards the alternatively activated and anti-inflammatory macrophage, with tumor-promoting capacities (also referred to as immune suppressive M2 phenotype), incapable of tumor cell killing, antigen presentation or T cell activation. This is due to TME-derived agents, such as IL-4, TGF-β and IL-10 that all favor an immunosuppressive TME (Figure 5). Thus, TAMs pick up a tumor-supporting phenotype, produce IL-10 and TGF-β by themselves, express Programmed death-ligand 1 (PD-L1) which suppresses T cell activation [215] and the production of TGF-β supports EMT of tumor cells that is needed for them to enter the circulation and metastasize [194,195]. Macrophages are known to uptake apoptotic cells but the uptake of apoptotic cells simultaneously diminishes their production of pro-inflammatory cytokines but instead supports anti-inflammatory cytokine production [216,217]. This suggests one possible way of immune evasion by tumor cells as like this the cytotoxic activity by TAMs against tumor cells is inhibited. Further, it has been shown that autoantibodies produced by B cells within the tumor drives TAM polarization and the engagement of activating FcγR promotes inflammation-associated tumor progression including tumor-angiogenesis [218,219]. New blood vessel formation as well as extracellular matrix remodeling further promotes cancer cell spread [202].

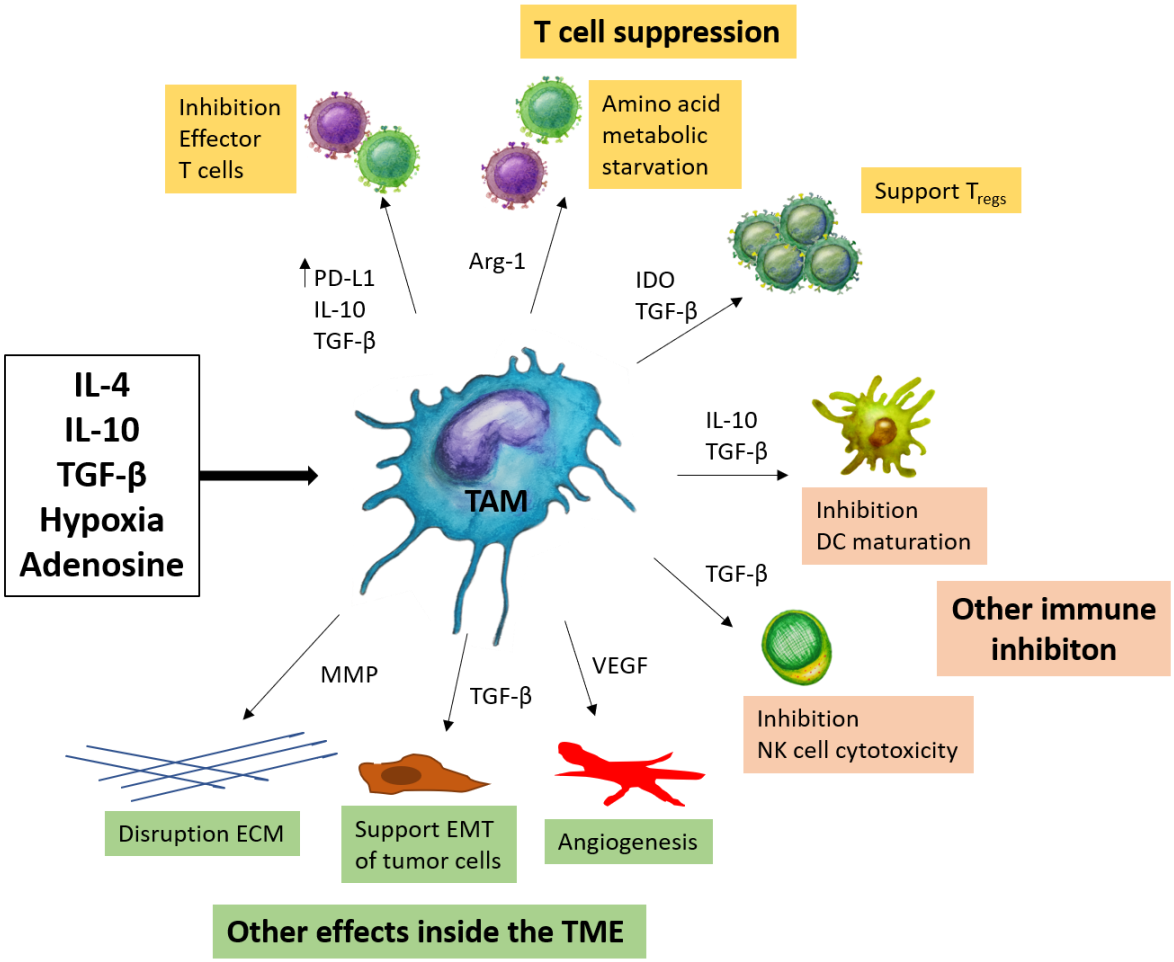


**Figure 5: Tumor-promoting functions of TAMs in the tumor microenvironment (TME).** Tumor-associated macrophages (TAMs) have various tumor-supporting functions in the TME. They are skewed towards an immune suppressive and pro-tumoral phenotype by tumor-derived mediators or produced by other cells inside the TME, such as IL-4, IL-10, TGF-β, adenosine or hypoxia. In turn, they upregulate a lot of immune inhibiting factors, such as IL-10 and TGF-β by which effector T cells, DCs and NK cells are inhibited to exert their anti-tumoral properties and regulatory T cells are instead supported. Disruption of extracellular matrix (ECM) by metalloproteinases (MMP), the support of epithelial to mesenchymal transition (EMT) of tumor cells and enhanced angiogenesis lead to increased tumor growth and risk of metastasis formation.

Recently, Yang and colleagues reviewed different TAM populations within the same tumor and how their tumor-supporting functions differ between their microenvironments. For this, they subdivided the tumor area into cancer cell invasion part, so-called "tumor nests" where the tumor cells are most dense, the hypoxic and the perivascular niche [214]. TAMs can support cancer cell invasion by the production of matrix metalloproteinases (MMP) for remodeling the extracellular matrix (ECM) and supporting tumor cell migration through disrupting basement membranes [214]. Moreover, the depletion of macrophages delayed the progression from preinvasive lesion to invasive carcinoma supporting the finding that these TAMs play a role in tumor cell invasion [220]. "Tumor- nests" are areas in the tumor defined by highest density of malignant cells. There is controversial data available whether high numbers of TAMs in these so called "tumor-nests" correlate with good or bad prognosis, it most probably depends on the type of cancer. For instance, high numbers correlate with good prognosis in endometrial cancer but with reduced prognosis in malignant melanoma [214]. The ECM consists mostly of non-malignant cells, such as immune cells, endothelial cells, pericytes and fibroblasts as well as

ECM molecules including collagen, fibronectin and laminin. Surprisingly, the TAM phenotype is also regulated by components of the ECM such as fibronectin and laminin and this finding is supported by another study that demonstrated decellularized ECM from human tumors polarize macrophages towards an immune suppressive phenotype with enhanced levels of IL-10 and TGF-β [221,222]. Hypoxic areas are characteristic for tumors, they have very little oxygen and are distant from tumor vessels and can be detected by staining for hypoxic cell markers, such as pimonidazole (PIMO) [214]. TAMs in hypoxic areas support blood vessel formation and express indoleamine 2, 3 – dioxygenase (IDO) that inhibits $CD8^+$ T cell proliferation and supports $T_{regs}$. Hypoxia induces a metabolic switch to increased glycolysis resulting in the accumulation of lactate within these areas. Lactate has been shown to differentiate TAMs into a proangiogenic subtype with enhanced VEGFA expression [123,214,223] and moreover, inhibition of lactate reduced the number of proangiogenic TAMs and tumor growth [223].

Perivascular TAMs support tumor angiogenesis, metastasis and are responsible for tumor relapse after therapies in some tumors. Furthermore, they are associated with markers that are expressed by M2 macrophages, such as MRC1 and CD163 and one specific receptor is Angiopoietin-1 receptor (TIE2), the main receptor for angiopoietins (protein growth factors required for the formation of blood vessels) [224]. These TIE2-expressing perivascular TAMs are also called "TIE2-expressing monocytes/macrophages" (TEM) and De Palma et al. reported the requirement of these TEMs for tumor vascularization by injecting $TIE2^+$ or $TIE2^-$ monocytes together with mouse mammary cancer cells. Tumors with $TIE2^+$ monocytes had increased vascularization compared to cancer cells alone or cancer cells injected with $TIE2^-$ monocytes [225]. The ligand of TIE2 is angiopoietin 2 (ANG2), an angiogenic factor expressed by activated endothelial cells and an increase in ANG2 supports tumor vascularization. The role of TEMs in tumor angiogenesis was confirmed by blocking TIE2/ANG2 signaling that decreased tumor vascularization dramatically and resulted in reduced tumor growth [226]. Also, tumor-infiltrating TEMs from mouse tumors showed higher expression of several M2-like tumor promoting genes compared to other macrophages from the same tumor, such as *Mmp9* and VEGFA [227] and the frequency of TEMs positively correlates with micro-vessel density in human cancers, such as HCC [228]. Furthermore, TEMs have been associated with interference of anti-cancer therapies and the relapse of primary lung and breast cancer mouse models after chemotherapy. These TEMs express VEGFA which stimulates re-vascularization and re-growth of cancer cells as well as high levels of CXCR4 which ligand is CXCL12. After chemotherapy, CXCL12 is upregulated in the perivascular niche, thus recruiting TAMs to the perivascular area. Blocking of CXCR4 decreased numbers of M2-like TAMs after chemotherapy, especially the ones in close proximity to blood vessels, thus decreasing re-vascularization and tumor re-growth [229]. Interestingly, this CXCR4/CXCL12 axis and the recruitment of new infiltrating monocytes to the perivascular areas has also been reported in untreated tumors [230]. When monocytes enter the tumor, tumor-derived TGF-β induces them to upregulate CXCR4 which then leads them through a chemotactic gradient to CXCL12-expressing fibroblasts close to tumor blood vessel. Once they arrive there they become metastasis-supporting perivascular TAMs [230].

Harney et al. revealed the presence of cells that were named "tumor microenvironments of metastasis" (TMEM) which consist of $TIE2^+$ $VEGFA^+$ TAMs that are in direct contact with cancer and endothelial cells. The tumor cells in TMEMs show increased motility due to the overexpression of the invasive isoform of the actin-binding protein mammalian enabled (MENA) and at the site of TMEMs there is enhanced intravasation of tumor cells into the vessels monitored [214,231]. In breast cancer patients, the frequency of TMEM positively correlates with the number of distant metastases compared to patients with only localized breast cancer [232].

## 1.6 CANCER IMMUNOTHERAPY

In some solid tumors, such as breast cancer, infiltrating immune cells can make up to 50% of the whole tumor [210]. Inflammatory cells are recruited to the tumor site and initially exert anti-tumor functions. However, the TME modulates the immune cells in a way that they become immune suppressive and support tumor progression. Therefore, immunotherapy is a way of harnessing the immune system to successfully fight malignant cells and eradicate the tumor. The aim is to unleash the immune system and reactivate innate and adaptive immune cells to carry out potent anti-cancer immune responses. This approach is an alternative to classic anti-cancer treatments such as chemotherapy where tumors can become resistant for.

Cancer immunotherapies are classified into active and passive therapy. The classification is based on the ability to (re-)activate the host immune system: Passive immunotherapy includes adoptively transferred T cells and the administration of tumor-targeting antibodies. Passive immunization does not induce memory responses but directly targets tumor cells or activates the immune system. In contrast, active immunotherapy includes anti-cancer vaccinations and checkpoint inhibitors. This leads to long-lasting anti-tumor immune responses and is called active because the engagement of the host immune system is required [233].

T cell-targeting immune checkpoint inhibitors, such as anti-PD-1, PD-L1 or CTLA-4 Abs, are helping many cancer patients to reduce their tumor burden and improved overall survival. However, some patients develop resistances against immunotherapies which is mostly mediated through tumor-associated myeloid cells, including macrophages [234] and additionally, not all patients respond fully to immunotherapy [235]. This can be due to escape mechanisms acquired by tumor cells, such as the loss of MHC-I and diminished antigen presentation [236]. This would then suggest implementing more NK cell-based immunotherapies which could sense these MHC-I deficient cells.

Targeting $MARCO^+$ TAMs as possible anti-cancer immunotherapy has been addressed in this thesis in paper II, III and IV. Anti-MARCO Abs were used as monotherapy but also in combination with other immune checkpoint inhibitors. Therefore, immune cell targeting by monoclonal Abs and other immunotherapeutic approaches will be discussed here.

### 1.6.1 Antibody-mediated immunotherapy

The use of monoclonal anti-MARCO Abs is a central topic of this thesis and the interaction with Fc receptors (FcR) is thematized in paper II and paper III, therefore, a short introduction is given here with specific focus on Fc γ receptors (FcγR).

FcγR control a variety of immunological functions in innate as well as adaptive immune cells by binding the Fc domain of IgG antibodies. The IgG subclass is the most common Ab type found in the blood and is produced by plasma cells after class-switching. IgG subclasses are in mice, IgG1, IgG2b and IgG3 and depending on the mouse strain either IgG2a (BALB/c) or IgG2c (C57BL/6); in humans, IgG1, IgG2, IgG3 and IgG4 and the affinity and specificity of therapeutic Abs to different Fc receptors dramatically mediates their effector function. The IgG subclass determines the mediated function, such as secretion of cytokines and chemokines, cellular activation, antigen uptake and presentation or the selection of B cells during their development. IgG Abs are Y-shaped proteins from which the "arms" are called Fab regions consisting of a light and a heavy chain as well as a constant and variable region. The variable region gives the Ab its antigen specificity and binds to the encountered antigen through its complementarity determining regions (CDRs). The lower part of the Y-shaped protein is called Fc part, binds to Fc receptors, consists of two heavy chains and mediates immune cell activity by generating appropriate immune responses dependent on the antigen.

FcR are divided into type I and type II FcR, depending on their binding stoichiometry and binding sites of the IgG Fc domain. Receptors of the type I group are related to the immunoglobulin receptor superfamily including FcγR, type II receptors belong to the family of C-type lectin receptors and include DC-SIGN (CD209) and CD23. For this thesis, type I FcR are of particular interest and will be described here in more detail.

Dependent on their intracellular signaling domain, type I FcγR are divided into activating or inhibitory receptors. In mice, there are three activating Fc receptors, FcγRI, FcγRIII and FcγRIV and one inhibitory Fc receptor FcγRIIb. This is different in humans who have five activating Fc receptors, FcγRI (CD64), FcγRIIa (CD32a), FcγRIIc (CD32c), FcγRIIIa (CD16a), FcγRIIIb (CD16b) but also only one inhibitory Fc receptor FcγRIIb (CD32b). Upon Ab binding, activating FcγR signal through immunoreceptor tyrosine activation motifs (ITAMs) in their cytoplasmic domain (FcγRIIa and FcγRIIc) or in the associated FcRγ chain (FcγRI, FcγRIIIa); in contrast, the inhibitory FcγRIIb transduces signals via the immunoreceptor tyrosine inhibition motif (ITIM) in its intracellular domain. Most of the hematopoietic cells express activating and inhibiting FcγR so the ratio of these will define the immunological response. Monocytes and macrophages express all FcγR whereas DCs express activating FcγRI and FcγRIII and the inhibitory FcγRIIb. B cells only express the inhibitory FcγRIIb and NK cells only the activating FcγRIII.

FcR have been implicated to be important in cancer therapy as they control Ab-dependent therapy responses. FcγRIIb-deficient mice are more sensitive to monoclonal Ab (mAb) therapy and show increased cytotoxicity as the inhibiting receptor has been depleted [237]. Furthermore,

FcγRIIb is required for agonistic activity of mAb immunotherapy targeting CD40 through crosslinking the receptors [238] but diminishes the efficacy of anti-CD20 mAb rituximab targeting B cells. As FcγRIIb is involved in the internalization of CD20/anti-CD20 complexes it inhibits ADCC of malignant B cells [239]. Furthermore, humanized FcR mice demonstrated the requirement of FcγRIIIa on macrophages for anti-CD20 mAb – mediated cytotoxicity [240]. Taken together, this demonstrates that for the design of new Ab based anti-cancer therapies the binding capacity of Fc parts to different FcR classes needs to be carefully evaluated.

Moreover, FcγR are also connected to macrophage polarization and activation: IL-4 has been reported to upregulate the inhibitory FcγRIIb receptor on M2 macrophages [85] and IFN-γ as stimulator for M1 macrophages downregulates FcγRIIb but promotes FcγRI expression on myeloid cells [241]. FcγRIIb-deficient macrophages show an increased inflammatory phenotype [242] and engaging activating receptors on macrophages by immune complexes leads to increased pro-inflammatory cytokine release [243]. However, the simultaneously stimulation of FcγRIIb inhibits the immune response [244], a modulatory pathway to avoid overactivated cells. Also, the ligation of activating FcγR in combination with TLR4 engagement leads to anti-inflammatory macrophage phenotype [245].

### 1.6.2 Targeting T cells

Immune checkpoint inhibitors were discovered for anti-cancer therapy to remove the brakes on T cells and to re-activate them. In 1996, James P. Allison demonstrated that Abs against the inhibitory receptor CTLA-4 on T cells suppresses tumor growth by promoting T cell reactivation and anti-tumoral properties [246]. This was the first time shown that CTLA-4 blocking can treat tumors in murine tumor models. However, it took another 15 years until the first anti-CTLA-4 Ab (ipilimumab) got approved by the Food and Drug Administration (FDA) as first immune checkpoint inhibitor for the treatment of stage IV melanoma patients in 2011 [247]. Anti-CTLA-4 Abs were the breakthrough in the immunotherapy field as for the first time immune-checkpoint blockade enhanced advanced – stage cancer patient survival. Ipilimumab was the first treatment that showed increased overall survival in advanced stage melanoma patients in a phase III clinical trial [248]. Other FDA approved immune checkpoint inhibitors are anti- PD-1 and anti-PD-L1 Ab.

CTLA-4 is expressed on activated T cells and works as a negative regulator. Upon the recognition of Ags in context of MHC complexes on APCs and the engagement of co-stimulatory receptor CD28, T cells get activated and upregulate their CTLA-4 expression. The inhibitory receptor CTLA-4 and the co-stimulatory receptor CD28 bind the same ligands, CD80 and CD86 expressed by APCs. Because CTLA-4 has a higher affinity than CD28, it dampens the T cell response and inhibits further T cell activation. Here is where anti-CTLA-4 Ab ipilimumab acts and suppresses this inhibiting signal resulting in more activated T cells [249]. CTLA-4 is exclusively expressed on T cells and regulates early T cell activation. Preclinial studies in mice showed that the success of anti-CTLA-4 Ab immunotherapy also stems from the depletion of $T_{regs}$ in the tumor [250,251]. However, a recent study by Sharma et al. shows that

this is not true for humans as a treatment with anti-CTLA-4 does not affect $FOXP3^+$ $T_{regs}$ numbers in human tumors [252].

Another inhibitory receptor on T cells is programmed cell death protein 1 (PD-1) which limits T cell activation in peripheral tissues. PD-1 is upregulated on T cells upon activation but can also be expressed by other cells, including NK cells and B cells [253]. Its ligands are Programmed death- ligand 1 (PD-L1, B7-H1) and PD-L2 (B7-H2) which are commonly expressed on APCs, such as DCs and macrophages, but also on cancer cells. PD-L1 is often overexpressed in tumors and can be found on immune cells within the TME, such as myeloid cells [253]. PD-L1 expression is enhanced in response to inflammatory signals including IFN-γ regulating T cell activation. PD-1/PD-L1 axis deals as negative feedback loop to prevent hyperactivation of T cells, reducing tissue damage and is crucial for maintaining peripheral tolerance [254]. Thus, PD-L1 expression by tumor cells inhibits T cell activation and suppresses their cytotoxic function by binding PD-1 on their surface which is blocked by PD-1 or PD-L1 targeted immunotherapy.

TAMs can also express both PD-1 and PD-L1 and PD-1 expression in TAMs has been negatively correlated with their capacity to phagocytose tumor cells. Hence, anti-PD-1 Ab treatment also targets TAMs and it has been shown that TAM – specific PD-1 blocking decreases tumor growth [255]. PD-L1 was also found on human TAMs in several cancer types, such as ovarian cancer and melanoma patients, and it positively correlates with the efficacy of anti-PD-1 Ab immunotherapy as monotherapy and in combination with anti-CTLA-4 Ab. Furthermore, PD-L1 expression on immune cells but not tumor cells is crucial for the efficacy of PD-L1 pathway blockade as shown in murine tumor models. Neither the knockout nor overexpression of PD-L1 in cancer cells affected the outcome of anti-PD-L1 directed therapy [256].

The combination of anti-PD-1 and anti-CLTA-4 Abs in melanoma and non-small cell lung cancer showed enhanced efficacy in treating cancers compared to monotherapies [257]. Therefore, many research groups try to find combinatorial treatments to further increase survival and success rates of immunotherapy.

### 1.6.3 Targeting tumor-associated macrophages

Tumor-associated myeloid cells represent the largest leukocyte population in tumors and TAMs became an emerging target for cancer immunotherapy during the past years. Several macrophage-targeted approaches are addressed to increase anti-cancer immune responses: The depletion of TAMs, diminishing their recruitment to the tumor site or exploiting their plasticity and repolarizing them from immune suppressive towards an inflammatory and tumoricidal phenotype.

The depletion of TAMs can be accomplished by inhibiting their replenishment by circulating monocytes or by inducing apoptosis of already present ones in the tumor. Inflammatory monocytes are recruited to the site of tumor by CCL2-CCR2 axis; therefore, blocking of CCR2 results in reduced TAM numbers, decreased tumor size and less metastasis [258,259] which in HCC has been shown to be $CD8^+$ T cell dependent [260]. Targeting the CCR2-CCL2 axis also improves

efficacies of immunotherapy [261] and radiotherapy [262]. In pancreatic cancer patients, the combination of CCR2 inhibition and chemotherapy enhanced tumor shrinkage by more than 40% compared to only chemotherapy [258]. However, this approach has downsides which need to be considered for designing future clinical trials: The interruption of CCL2 blockade released monocytes into the circulation that were previously trapped in the bone marrow which in turn increased metastases and accelerates deaths in a murine breast cancer model [263]. Another way of depleting TAMs is to target the CSF1-CSFR1 axis which results in apoptosis of a substantial part of TAMs in most tumors. Inhibition of the CSF1 and CSFR1 pathway improves the efficacy of immunotherapies, such as anti-PD-1 Ab and anti-CTLA-4 Ab, CD40 agonists and enhances T cell responses when combined with chemotherapy or radiation [264]. The disadvantage of targeting this pathway is the depletion of other tissue-resident macrophages which are needed for tissue homeostasis and the removal of apoptotic cells. Also, depletion of TAMs leads to the loss of TAM-derived IL-15 and thus to lower numbers of NK cells in the tumor and increased metastasis formation. The administration of IL-15 to mice that were treated with CSF1R-inhibitor rescued NK cell numbers and attenuated metastasis [265].

Recruitment of monocytes that eventually differentiate into TAMs can be modified by addressing the CXCR4-CXCL12 and ANG2-TIE2 axis. Treatment resistance and tumor regrowth after chemotherapy and radiation is connected with increased levels of CXCL12 in breast cancer and glioma which supports the repopulation of CXCR4-expressing TAMs [229,266]. CXCL12 recruits mainly $TIE2^+$ TAMs that strongly support tumor vascularization [226] and the inhibition of TIE2 results in reduced tumor growth, less metastases in breast cancer [267] and decreased angiogenesis [226]. The combination of neutralizing ANG2 and blocking VEGFA by bispecific Abs enhances the efficacy compared to single treatments and enhances T cell infiltration. In response, perivascular T cells upregulate PD-L1 and the anti-tumor response of ANG2 and VEGFA blockade could be further increased with anti-PD-1 Ab treatment [268].

By depleting TAMs, their tumoricidal capacity and the potential to elicit immune stimulatory functions as phagocytes and APCs within the tumors is also gone. Therefore, many studies approach to repolarize the immune suppressive and pro-tumorigenic TAMs towards a pro-inflammatory phenotype with anti-tumor activity. One candidate is CD40, a member of the tumor necrosis factor (TNF) receptor super family and crucial for the co-stimulation of immune cells. It is expressed on APCs, as DCs, macrophages and B cells but also on other cells including epithelial and endothelial cells. Its ligand CD40L is predominantly expressed by activated $CD4^+$ T cells. Upon binding of CD40 and CD40L, macrophages upregulate co-stimulatory and Ag presenting molecules and release pro-inflammatory cytokines, such as IL-12 and TNF- α. IL-12 in turn activates NK cells and enhances T cell activation. Agonistic anti-CD40 Abs showed reduced tumor growth and improved overall survival in pancreatic cancer in combination with chemotherapy and also αCD40/chemotherapy combined with αPD-1 and/or αCTLA-4 [269]. Also the combination of CSF-1R antagonists and CD40 agonistic Abs leads to TAM polarization, increased pro-inflammatory cytokine production and $CD8^+$ T cell activation and subsequently decreased tumor growth and improved survival in different mouse tumor models [206].

As the ligation of TLRs on macrophages activates them towards a pro-inflammatory phenotype, TLR agonists have been developed to target TAMs and TLR7, TLR8 and TLR9 agonists show promising results by increasing macrophage repolarization and enhancing tumoricidal activities in breast cancer and melanoma models [210].

Tumor cells can evade immune cell recognition by expressing specific "don`t eat me" signals to suppress the phagocytic capacity of macrophages. Normally, CD47 is expressed on host cells and upon binding to its ligand SIRP-α on macrophages it mediates a "don`t eat me" signal protecting the host cell against being phagocytosed. However, tumor cells can acquire CD47 and binding of CD47 to SIRP-α results in the inhibition of TAMs. CD47 is overexpressed in many tumors [210] and anti-CD47 blocking Abs reduce tumor growth in several tumor models. Furthermore, they are currently tested in clinical trials as single agent or in combination with cetuximab, an inhibitor of epidermal growth factor receptor (EGFR) [206].

Also, targeting on molecular level has been reported to successfully reprogram TAMs. The activation of phosphoinositide 3-kinase γ (PI3Kγ) in macrophages has been shown to skew TAMs to the immune suppressive phenotype and blocking PI3Kγ repolarizes TAMs including increases $CD8^+$ T cell mediated anti-tumor response in PDAC [234,270]. The blocking of PI3Kγ signaling enhances the production of pro-inflammatory genes in macrophages in a NFκB – dependent manner and results in tumor decrease in murine lung, pancreas, breast, melanoma and head and neck squamous-cell carcinoma (HNSCC) [206,234]. Combining PI3Kγ inhibitors with anti-PD-1 Abs improved survival and further reduced tumor growth in mice with HNSCC [234]. In myeloid cells, PI3Kγ is also crucial for the recruitment to tumor sites as it is activated downstream of many chemoattractant receptors in mice and humans. Its activation leads to conformational changes in integrin alpha 4 beta 1 (VLA-4) which then binds the vascular cell adhesion molecule (VCAM-1) promoting extravasation of monocytes into the tumor. Therefore, PI3Kγ inhibition results in less myeloid cells in the tumor and reduces tumor growth as well as metastases in murine breast, pancreatic, lung and melanoma tumors [206].

The targeting of TAMs by monoclonal Abs is investigated in paper II, III and IV. We target scavenger receptor MARCO on immune suppressive TAMs and repolarize them towards a pro-inflammatory phenotype.

### 1.6.4 Targeting natural killer cells

NK cells are professional killers of malignant cells and they also interact with other immune cells to execute a potent anti-tumor response. In mice, NK cells are identified as $CD3^-$ $NKp46^+$ (in BALB/c mice) or as $CD3^-$ $NKp46^+$ $NK1.1^+$ (in C57BL/6 mice). In humans, it is distinguished between immature $CD3^-$ $CD56^{bright}$ $CD16^-$ and mature $CD3^-$ $CD56^{dim}$ $CD16^+$ NK cells [271,272]. Immature NK cells are more susceptible to activation through cytokines IL-2, IL-15 and IL-12 and subsequent production of IFN-γ while mature NK cells comprise more granules to rapidly elicit cytolytic function, containing cytolytic molecules such as granzymes and perforin [272]. 90% of blood-circulating NK cells are of mature phenotype [273]. Once NK cells get activated, they form immune synapses with target cells and release their preformed cytolytic

granules to lyse the cell. However, there are limitations for NK cells and NK cell-based immunotherapies based on their poor ability to infiltrate tumors and the immunosuppressive TME that inhibits NK cell cytolytic activity through different factors, such as TGF- β or adenosine. Additionally, tumor cells adapt and escape NK cell recognition by downregulating ligands for activating NK cell receptors or reducing MHC-I expression on their surface [271].

NK cells can exert their cytolytic function in tumors in different ways including the release of cytotoxic molecules by sensing the "missing- self" HLA-1 expression, by the engagement of death receptors (DR) and by antibody dependent cell-mediated cytotoxicity (ADCC) – this broad cytotoxicity makes NK cells perfect candidates for cancer immunotherapy.

Binding of DRs expressed by NK cells, such as Fas ligand and TRAIL, to their ligands on target cells will induce apoptosis through a caspase-dependent enzymatic cascade. Interestingly, TRAIL receptors can also be expressed by monocytes and TAMs and upon treatment with recombinant TRAIL these cells specifically undergo apoptosis in a caspase 8-dependent manner [274]. TRAIL-deficient mice are more susceptible to tumor growth and metastases and TRAIL-neutralizing Abs promote tumor progression suggesting TRAIL as an important player in anti-tumor responses [275]. Recombinant versions of TRAIL and agonistic anti-TRAIL receptor Abs are currently in development [275].

Another way of tumor cell killing by NK cells is ADCC mediated by IgG Abs binding to tumor cells. The Fc part is recognized by the activating Fc receptor FcγRIIIa on NK cells resulting in the release of cytolytic granules to kill the target cell [271]. IgG1 and IgG3 Abs have the highest affinity for FcγRIIIa mediating tumor cell killing and clinically used Abs with increased Fc receptor affinity are developed at the moment. There are bi- or tri-specific killer cell engagers (BiKEs or TriKEs) that are against tumor-associated antigens and activating NK cells receptors to initiate an immunologic synapse and induce tumor cell killing more efficiently. Moreover, NK cell survival and activation factor IL-15 can be targeted for cancer immunotherapy and the administration of recombinant human IL-15 supported proliferation of NK cells and $CD8^+$ T cells [276]. NK cells also express inhibitory receptors that can deal as immune checkpoints including KIRs, T cell immunoreceptor with Ig and ITIM domains (TIGIT) and PD-1. Recently, it has been shown by Zhang et al. that the blocking of checkpoint receptor TIGIT prevents NK cell exhaustion and leads to a highly efficient NK cell-mediated tumor-specific T cell response [277]. NK cells recruit DCs to solid tumors and activate them through IFN-γ production correlating with improved survival of cancer patients.

Another promising approach is the adoptive transfer of allogeneic NK cells that have been activated *ex vivo* or chimeric antigen receptor (CAR)- modified NK cells. CARs bind tightly to tumor specific antigens mediating NK-tumor cell interaction and their intracellular domain containing ITAMs activate the NK cell. To further increase NK cell function, the intracellular domain can also contain co-stimulatory protein receptors [271]. CAR- NK cells can be derived from PBMCs, NK cell lines and hematopoietic stem cells which makes them more attractive then CAR- T cell therapy because these are required to be autologous.

# 2 THE PRESENT STUDY

## 2.1 AIMS

This thesis aims to investigate the role of scavenger receptor MARCO in immune responses as well as its potential as target for cancer immunotherapy.

**Paper I** – To study the effect of targeting scavenger receptor MARCO on marginal zone macrophages by monoclonal antibodies and the subsequent immune response.

**Paper II** – To examine the impact of targeting scavenger receptor MARCO on tumor-associated macrophages by monoclonal antibodies and modulating the immune suppressive tumor microenvironment.

**Paper III** – To investigate the mechanism of how anti-MARCO antibody treatment leads to decreased tumor burden in melanoma.

**Paper IV** - To modulate human immune suppressive $MARCO^{+}$ myeloid cells to enhance the tumorigenic phenotype of immune cells in pancreatic cancer by engaging MARCO.

## 2.2 RESULTS AND DISCUSSION

### 2.2.1 Marginal zone macrophages regulate antigen transport by B cells to the follicle in the spleen via CD21 (Paper I)

MARCO expressing MZMs aligning the marginal sinus in the spleen are positioned strategically to filter antigens and pathogens from the circulation. It has previously been shown that SLE patients have autoantibodies against scavenger receptor MARCO but their role in the disease and impact on other immune cells is not known [44]. Therefore, in this study we address the impact of anti-MARCO Abs on MZMs and the interaction between MZMs and the closely located MZBs as well as on the subsequent immune response towards foreign Ag.

To investigate the impact of anti-MARCO Ab on the MZM/MZB interaction, we injected rat anti-mouse MARCO Abs intravenously (i.v.) in wildtype (wt) mice. We confirmed that we specifically targeted MARCO$^+$ MZMs without depleting them and this resulted in the gradual loss of complement receptor 2 (CD21) on MZBs. Already 1 hour after Ab injection we noticed a reduction of CD21 on MZBs; other B cell populations were also affected but to a lesser degree. Further analysis excluded local complement activation or receptor internalization to be responsible for the reduction in CD21. Next, we investigated whether the alternative splice – variant CD35 (complement receptor 1) was affected and monitored that it was also reduced on MZBs to the same extent as CD21; however, leaving the CD35 expression on FDCs unaffected. Polyclonal Ab serum from mice immunized with MARCO protein also led to the reduction of CD21 on MZBs ruling out any inter-species effect. Moreover, as MARCO can also be upregulated on macrophages upon binding of bacterial Ag, such as LPS, we analyzed MyD88-, TLR2-, TLR4- and TLR9- deficient mice and found none of the molecules were involved in mediating the reduction in CD21 on MZBs after anti-MARCO Ab administration. Also, we excluded any assistance of FcγRs.

The fact that CD21 on MZBs is so quickly reduced after anti- MARCO Ab injection led us to the hypothesis that CD21 might be cleaved upon MARCO engagement. It is known that CD21 and adhesion molecule L-selectin CD62L can be shedded from B cells [278]. Indeed, we found a similar decrease of CD62L on MZBs after MARCO engagement as CD21. Purinergic receptors, such as P2X7R, are known to cleave CD21 and CD62L in response to extracellular ATP and soluble CD21 has been found linked to different inflammatory responses and autoimmune diseases [278]. Therefore, we set up *in vitro* experiments to analyze the mechanism underlying the decrease in CD21, CD35 and CD62L on MZBs after anti-MARCO Ab injection. Using MARCO$^+$ peritoneal macrophages, we detected the release of extracellular ATP already 5 minutes after Ab stimulation (Figure 6). We

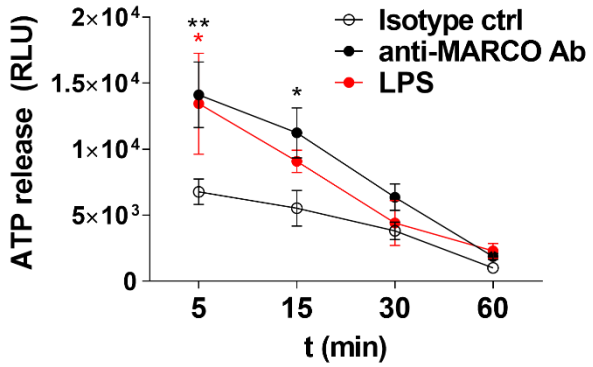


**Figure 6: The release of extracellular ATP after anti-MARCO Ab treatment.** Peritoneal macrophages were stimulated and analyzed *in vitro*.

confirmed that the loss of CD21 and CD62L on the surface of MZBs is dependent on extracellular ATP using cultures of splenocytes *in vitro*. Co-culture experiments of $MARCO^+$ macrophages and splenocytes revealed that the anti-MARCO Ab mediated CD21 decrease could be reversed by adding ATPase. Together, this strengthened our hypothesis that macrophages mediated ATP release is connected to the loss of CD21. To confirm this *in vivo*, we injected ATP i.v. which mimicked the reduction of CD62L on MZBs that we saw *in vitro* but not CD21. This could be because of differences in receptor expression or cleavage efficacy *in vivo*. To further delineate the involvement of extracellular ATP we analyzed the expression of ectoenzyme CD39 which is an important player in the conversion from extracellular ATP to adenosine which in turn is known for its immune modulatory and immune suppressive capacities [279]. Interestingly, ectonucleotidase CD39 was upregulated on macrophages upon 24 hours of anti-MARCO Ab stimulation *in vitro* to similar extent as after the addition of extracellular ATP. Normally, extracellular ATP acts as a danger signal and can program macrophages towards a pro-inflammatory phenotype [170] as well as activate other immune cells. Thus, it is important to have modulatory mechanisms to prevent over activation by upregulating molecules, such as ectonucleotidases. These enzymes convert ATP to AMP and to the immunomodulatory adenosine which in turn suppresses immune cells [279]. To further explore the involvement of the purinergic receptor P2X7R, we injected P2X7R- deficient mice with anti-MARCO Ab or vehicle i.v. *in vivo* or added ATP to the cultures of P2X7R- deficient splenocytes and we still saw a reduction of CD21 and CD62L suggesting that other ATP-driven mechanisms must be responsible for the loss of these receptors.

Next, we investigated whether any functions of MZBs are impaired by the CD21 loss as MZBs are important players for a potent immune response. In the spleen, MZBs migrate between marginal zone and follicle to transport antigen and deliver it to FDCs. Targeting MARCO on MZMs did not interfere with the shuttling of MZBs to the follicle but it reduced the transport of CD21- dependent Ag to the follicle resulting in less Ag deposition on FDCs after 24 hours. Scavenger receptor MARCO is known to bind self-Ags and the mechanism we discovered could be a modulatory mechanism to prevent the transport of these self-Ags to the follicle inducing an autoimmune response. The deposition of Ag on FDCs is important for the activation of germinal center formation and the subsequent immune response. Thus, we investigated the humoral response and found less GC B cells as well as reduced IgM, IgG1, IgG3 titers in response to a T cell – dependent Ag and less IgM to T cell – independent Ag. Considering the decreased Ag transport and lower Ab levels upon anti-MARCO Ab injection, it is possible that the anti-MARCO autoantibodies found in SLE patients are one factor for the increased risk for inflammation in these patients.

Taken together, this study reveals a new mechanism of how MZMs in the spleen can regulate the adaptive and humoral immune response. Anti-MARCO Ab injection leads to the release of extracellular ATP by MARCO expressing macrophages which in turn results in the loss of CD21 and CD62L on MZBs. Moreover, CD21- dependent Ag transport by MZBs into the follicle and Ag deposition on FDCs is reduced after anti-MARCO Ab injection and the subsequent humoral response is impaired. Our findings go in line with that SLE patients also

show lower levels of CD21 on B cells and it will be interesting to investigate this further to find out if it is possible to target that pathway to modulate Ag transportation into the follicle to prevent unwanted immune responses.

### 2.2.2 Reprogramming tumor-associated macrophages by antibody targeting inhibits cancer progression and metastasis (Paper II)

Tumors are diverse tissues that consist of many different cell types besides malignant cells. Immune cells play an important role in the TME and inflammation has been identified as one of the hallmarks of cancer [181]. Especially tumor-associated macrophages (TAMs) as a very plastic cell type can contribute to tumor growth in different ways: They can enhance tumor progression by supporting angiogenesis, metastasis formation or the suppression of other immune cells; however, they can also show inflammatory properties by the release of extracellular ATP activating other effector cells or the expression of pro-inflammatory cytokines. TAMs are present in nearly all tumors and they are known to play a role in maintaining an immune suppressive TME [206].

So far, the expression of scavenger receptor MARCO has been thought to be restricted to some macrophage subpopulations, such as alveolar, peritoneal or marginal zone macrophages that we targeted in paper I. However, we found MARCO also expressed on TAMs in several mouse tumor models, including melanoma, mammary adenocarcinoma and colon adenocarcinoma. We identified MARCO to be exclusively co-localized with the macrophage marker F4/80 and not expressed on other immune cells. However, not all $F4/80^+$ cells were positive for MARCO: By sorting different TAM subpopulations we detected $CD45^+$ $CD11b^+$ $Ly6G^-$ $Ly6C^{low}$ $MHCII^{low}$ cells as the MARCO expressing macrophages. MARCO expression correlated with high expression of typical genes that are expressed by anti-inflammatory macrophages, such as *arg1*, *fizz1* and low expression of genes that relate to inflammation such as *Tnf*, *Il-1b or Nos2*. This suggested that MARCO is a marker for an anti-inflammatory and immune suppressive TAM subset. To further investigate the $MARCO^+$ subpopulation and what drives MARCO expression, we set up an *in vitro* system using bone marrow-derived macrophages (BMDMs) which we polarized towards the pro-inflammatory (IFN-γ + LPS) or immune suppressive (IL-4 + IL-13) phenotype as well as incubated with B16 melanoma cell line-conditioned medium. Our findings *in vitro* go in line with the *in vivo* data and we confirmed that MARCO is expressed on the immune suppressive subtype and can be induced by tumor-conditioned medium. As the cytokines IL-10 and TGF-β are prominent immune suppressive cytokines in the TME and they can polarize TAMs towards an anti-inflammatory phenotype, we evaluated if they also have the capacity to induce MARCO expression and both cytokines certainly upregulated MARCO *in vitro* on a peritoneal macrophage cell line.

Previously, we have shown that targeting MARCO with monoclonal Abs influences the immune response by modulating Ag shuttling and Ab production (paper I), thus, we analysed if targeting $MARCO^+$ TAMs also changes the anti-tumor immune responses. For this, we injected a rat anti-mouse MARCO Ab i.v. and monitored tumor growth in the B16 melanoma and 4T1 breast adenocarcinoma model. We confirmed that the anti-MARCO Ab infiltrated the

tumor and reached the MARCO expressing cells. Interestingly, anti-MARCO Ab treatment led to decreased tumor growth in both tumor models and in the 4T1 mammary carcinoma also to less metastasis formation to the lungs (Figure 7). Furthermore, we noticed an increase in pro-inflammatory and a decrease in immune suppressive TAMs in the 4T1 model as well as an altered $CD4^+/CD8^+$ T cell ratio, less regulatory T cells within the tumor and more GC B cells in the draining lymph node of tumor-bearing mice.

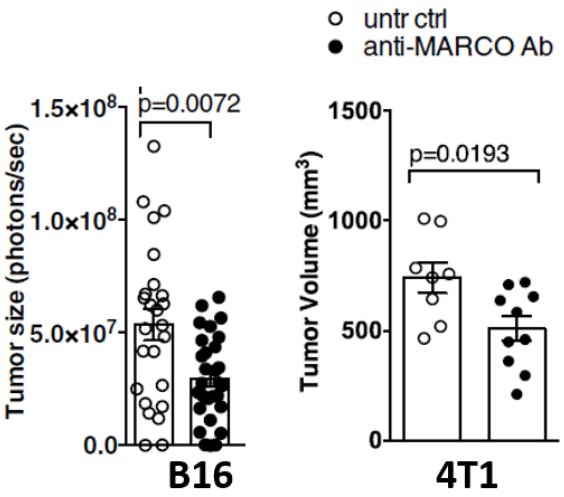


**Figure 7: Targeting MARCO by anti-MARCO Ab reduces tumor growth *in vivo*.** Tumor volume in B16 melanoma (day 12, left) and 4T1 mammary carcinoma (day 21, right) tumor models in control and anti-MARCO Ab treated mice. Mice were inoculated with tumor cells subcutaneously and treated with anti-MARCO Ab intravenously.

To further investigate the properties of anti-MARCO Ab treatment as anti-cancer immunotherapy, we used the B16 melanoma model. Tumor cells were injected subcutaneously and tumor growth was inhibited by anti-MARCO Ab treatment to the same extent as with the TA99 Ab which is known to induce ADCC to B16 tumor cells. By sorting bulk TAMs and analysing their gene expression, we observed increased expression of pro-inflammatory genes in the anti-MARCO Ab treated mice compared to the control group, such as *Il1β*. Moreover, TAMs from the treated group also showed a decrease in anti-inflammatory genes, such as *il10*, suggesting the reprogramming from immune suppressive to a pro-inflammatory macrophage subtype mediated by anti-MARCO Ab administration. Analysis by flow cytometry revealed that the ratio of intratumoral $CD4^+/CD8^+$ T cells was altered, numbers of $T_{regs}$ decreased and OVA – specific $CD8^+$ T cells were increased. This goes in line with increased OVA- specific IgG2b levels in the serum of anti-MARCO Ab treated mice.

Harnessing the potential of the immune system is already implemented and immune checkpoint inhibitors targeting T cells show promising results in patients of several cancer types. However, these immunotherapies still have limitations and do not work for all patients. Therefore, we assessed the capacity of combining the anti – MARCO Ab treatment with the checkpoint inhibitor anti-CTLA-4. Interestingly, the combination of these two mAb treatments increased their efficacy compared to single treatments resulting in less tumor growth in the B16 melanoma as well as the MC38 colon adenocarcinoma model.

It has been shown that anti-tumor Abs require the engagement of FcR to be functional either through promoting cytotoxicity or through crosslinking of the receptors and thus inducing agonistic capacity [238]. Therefore, we evaluated the involvement of FcR in the anti-MARCO Ab mediated anti-tumor effect. For this, we generated anti-MARCO Ab variants with modified Fc domains or used mice that were either deficient for all FcR, only the activating FcγRI, III or IV or only missing the inhibitory FcγRIIb. Consequently, we observed that the therapeutic anti-

tumor effect mediated by anti-MARCO Ab was dependent on the inhibitory FcγRIIb which we also found to be highly expressed on MARCO$^+$ TAMs *in vivo* and on *in vitro* derived MARCO$^+$ TAMs.

Additionally, to translate our findings to humans we next investigated MARCO expression in human breast cancer and metastatic melanoma biopsies. We had access to the The Cancer Genome Atlas (TCGA) and KI/Clinseq databases and observed the highest MARCO expression in the basal (triple negative) subgroup of breast cancer patients and for melanoma patients in distal metastases. Taken together, in both tumor types MARCO expression correlates with the more aggressive and metastatic cancer subtypes. In support with our findings in mice, MARCO expression was also in humans associated with immune suppressive macrophage markers such as *il4r*, *retnlb* and *cd163* and FcγRIIb and furthermore, with an EMT gene signature expressing the known EMT regulators *mmp9*, *snai1* and *twist1* indicating a metastasis-promoting role.

Finally, we validated MARCO expression on human tumor sections from breast cancer and melanoma patients by immunofluorescence and confirmed that MARCO is co-expressed with macrophage marker CD68$^+$ and that it also strongly correlates with CD206 and CD163 which are both markers for immune suppressive macrophages in humans. Furthermore, triple negative breast cancer patients had the highest infiltration of CD68$^+$ TAMs. These findings strengthen our hypothesis of MARCO being a marker for the immune suppressive macrophage subpopulation also in humans.

In summary, this study uncovered scavenger receptor MARCO as a marker for the immune suppressive TAM population and revealed it as novel candidate for targeted immunotherapy using Abs. MARCO is also expressed in clinical samples of human breast cancer and melanoma patients where it correlates with anti-inflammatory and metastatic genes. Targeting MARCO with mAbs in pre-clinical mouse models showed impressive decrease of tumor growth in primary as well as metastatic tumors in several cancer models. This is due to re-programming of immune suppressive macrophages towards a pro-inflammatory phenotype with increased anti- tumor immunogenicity (Figure 8). The combinatorial treatment of anti-MARCO Ab and checkpoint inhibitor anti-CTLA-4 Abs resulted in further decrease in tumor size compared to single treatments. Altogether, these data suggest that TAMs can be targeted with Abs to modulate the immune suppressive TME by activating immune cells to increase their immunogenicity.

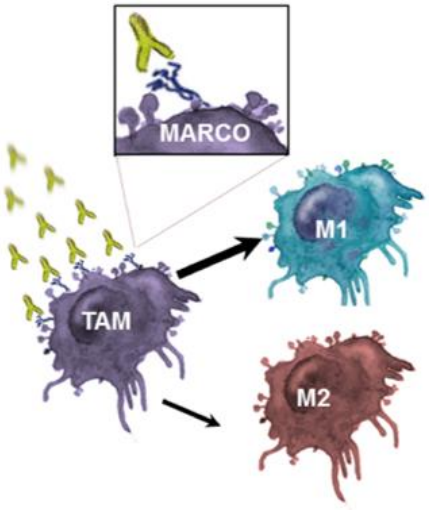


**Figure 8: Reprogramming of immune suppressive to pro-inflammatory TAMs by targeting MARCO with antibodies.** From reference #80.

### 2.2.3 Targeting scavenger receptor MARCO on tumor-associated macrophages activates TRAIL-dependent tumor cell killing by NK cells in melanoma (Paper III)

Immunotherapy is a promising approach for anti-cancer therapy and with checkpoint inhibitors anti-CTLA-4, anti-PD-1 and anti-PD-L1 Abs there are already some successfully used in the clinics. Most of the FDA-approved immunotherapies target T cells but as we have shown recently, also TAMs can be targeted by Abs (paper II). Thus, we re-program immune suppressive and tumor-supporting macrophages towards a pro-inflammatory phenotype with tumoricidal capacities. Based on this, we wanted to understand the impact of anti-MARCO Ab treatment on the TME and how it results in reduced tumor growth.

In order to elucidate the mechanism of anti-MARCO Ab treatment resulting in smaller tumors, we investigated the $MARCO^+$ TAMs in more detail. Interestingly, in the B16 melanoma model they showed a perivascular macrophage phenotype with high expression of Tie2 and CXCR4 [229,280] and were in close proximity to $CD31^+$ blood vessels. Performing whole tumor imaging, we revealed that anti-MARCO Ab treatment decreased the tortuosity of $CD31^+$ vessels with a trend to reduced numbers of nodes but vessel radius and volume were not affected. This suggests that targeting MARCO does not have a profound effect on tumor vascularization but affects it partly thus leading to tumor reduction.

Further investigating the events mediated by MARCO engagement, we found scavenger receptor MARCO to be internalized upon Ab binding which gradually increases with time and is partly dependent on FcR. It is still not known how MARCO exactly signals but it has been shown that class A scavenger receptors are internalized upon ligand binding [147] which strongly suggest that MARCO has a similar signaling pathway.

We have previously shown that targeting MARCO induced the release of extracellular ATP by macrophages (paper I); therefore, we evaluated whether the anti-MARCO Ab mediated anti-tumor effect is dependent on the ATP-sensing receptor P2X7R. P2X7R is an ionotropic receptor that forms pores upon ligand (ATP) binding. The binding of ATP leads to conformational rearrangements allowing transmembrane fluxes needed for the activation by P2X7R [16]. Running the B16 melanoma model in P2X7R–deficient mice validated that this receptor is indeed needed for the anti-tumor response by MARCO targeted therapy. P2X7R can activate the NLRP3 inflammasome and thus lead to a pro-inflammatory phenotype; however, tumor studies in NLRP3-deficient mice elucidated that anti-MARCO Ab treatment does not require NLRP3 to reveal its tumorigenic properties. Another way of action would be that P2X7R results in metabolic changes within the cells. And indeed, after anti-MARCO Ab treatment, we detected that $MARCO^+$ macrophages changed their metabolomics including increased glycolysis and *Hif1α* expression *in vitro*. Augmented glycolysis and HIF-1α levels are known to be associated with pro-inflammatory macrophage phenotype [121,281] which further supports our findings from paper I and II.

To assess the role of other immune cells in the anti-tumor effect mediated by anti-MARCO Ab treatment, we depleted different effector cells in the B16 melanoma model, in particular $CD4^+$ or $CD8^+$ T cells or NK cells. We injected depleting Abs 24 hours before tumor cell inoculation and administered the anti-MARCO Ab every third day. Surprisingly, the anti-MARCO Ab immunotherapy in the melanoma model was completely independent on T cells but dependent on NK cells (Figure 9).

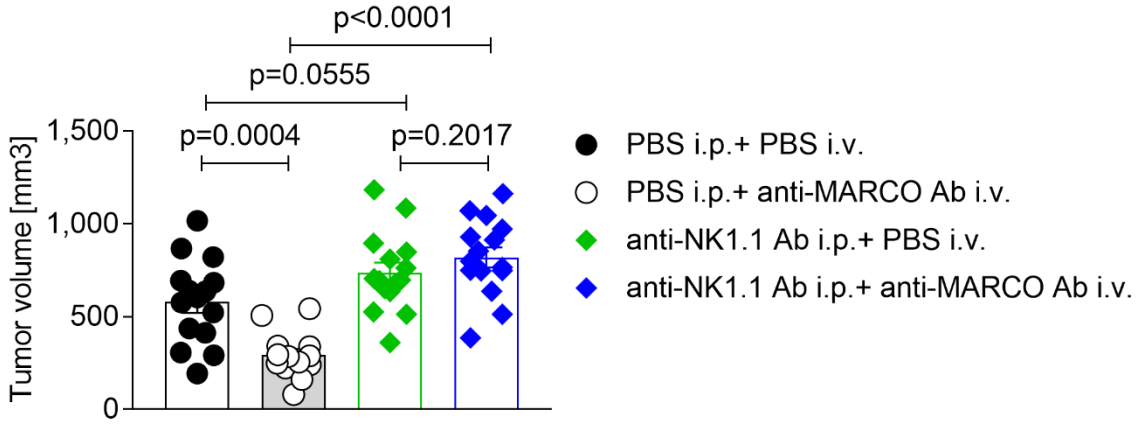


**Figure 9: Anti-tumor effect mediated by anti-MARCO Ab treatment is dependent on NK cells in the B16 melanoma model.** Tumor volume is shown on day 10 and in WT mice we see smaller tumors in the treated group (empty dots) compared to PBS control group (black dots). However, when NK cells were depleted this decrease in tumor size upon anti-MARCO treatment (blue dots) is gone compared to PBS group (green dots). Mice were injected with NK cell depleting Abs or vehicle 24 hours before subcutaneous tumor cell inoculation and then treated with anti-MARCO Ab every third day.

Also, immunofluorescence stainings of tumor sections verified $MARCO^+$ cells closely located to NK cells. However, we could not detect any differences in the expression of NK cell maturation or activation markers after anti-MARCO Ab treatment, as NKG2D, DNAM1, KLRG1 expression measured by flow cytometry. Interestingly, we discovered a decrease of ectoenzyme CD39 on NK cells after the Ab injection. As described before, CD39 is involved in the conversion from extracellular ATP to AMP which is then further converted to adenosine by CD73 [279]. Adenosine has immune suppressive capacities and reduces NK cell cytotoxicity, so the decrease of CD39 might be a way to regulate the levels of immune suppressive adenosine in the area and to keep the NK cells more activated. This strengthens our hypothesis that targeting MARCO by Ab creates a more immunogenic TME. Additionally, we detected higher levels of IL-15 in the serum of tumor-bearing anti-MARCO Ab treated mice which is known to activate NK cells. As our anti-tumor effect is Ab-mediated, we ruled out that NK cells deplete $MARCO^+$ immune suppressive TAMs by ADCC using FcγRIII-deficient mice. We found increased levels of the effector molecules perforin and TRAIL on intra-tumoral NK cells after anti-MARCO Ab treatment and confirmed that our B16 melanoma cell line expresses the TRAIL ligand DR5. By blocking TRAIL *in vivo*, we observed that the MARCO-directed immunotherapy needed the TRAIL-dependent killing mechanism to work but not perforin as tumor experiments in perforin-deficient mice demonstrated.

Additionally, the effect of MARCO targeting immunotherapy in combination with the immune checkpoint inhibitors anti-PD-1 and anti-PD-L1 Abs has been evaluated. These checkpoint

therapies are promising and show impressive results in the clinics; however, not all patients are responding to single therapies which is why many studies try to find combinatorial treatments. With anti-PD-1 and anti-PD-L1 Ab mainly targeting T cells and anti-MARCO Ab directed at TAMs, we combined these immunotherapies. The combination of anti-MARCO Ab with either one of the checkpoint inhibitors increased their efficacy compared to single treatments. Furthermore, we detected increased NK cell infiltration of the tumor but no dramatic differences in other immune cells.

To translate our findings and the connection between anti-MARCO Ab treatment and NK cells to humans, we set up an *in vitro* system to establish human MARCO expressing macrophages from $CD14^+$ monocytes. We used IFN-γ + LPS for pro-inflammatory macrophages, IL4 + IL-10 for immune suppressive macrophages and in addition, conditioned medium from three different human primary melanoma cell lines (ANRU, KADA and MAT02) to polarize the macrophages. We previously showed that MARCO is expressed on immune suppressive TAMs in several human cancers including melanoma patients (paper II) and as expected MARCO expression was induced by IL-4 + IL-10 and the melanoma cell-conditioned media.

To address the human MARCO receptor, we produced anti-human MARCO Ab by immunizing mouse MARCO-deficient mice with the human MARCO protein. We then performed co-culture experiments of human NK cells and macrophages in tumor-conditioned medium which blocked NK cell activation compared to normal medium, measured by IFN-γ production, proliferation measured by Ki67 and degranulation analyzed by CD107a *in vitro*. However, when macrophages were pre-treated with anti-hMARCO Ab we were able to re-active the NK cells by restoring activation, proliferation and degranulation capacity.

Finally, we assessed the impact of anti-hMARCO Ab treatment on the killing capacity of NK cells towards the primary human melanoma tumor cell lines KADA and ANRU. For this, NK cells were co-cultured with macrophages in tumor–conditioned medium which decreased the killing ability by NK cells compared to normal medium. However, when adding anti-hMARCO Ab to the cultures, we rescued NK cell activation resulting in enhanced killing.

Overall, our results show for the first time that targeting immune suppressive TAMs by mAbs leads not only to re-programming of TAMs towards a pro-inflammatory and more tumorigenic phenotype but that MARCO directed immunotherapy also activates NK cells for increased TRAIL-mediated tumor cell killing. This mechanism also holds in *in vitro* assays with human cells which is promising for the future to bring anti-MARCO Abs into the clinics. Current immunotherapies mostly target and activate T cells but it is important to identify combinatorial treatments to not only target one single cell type to enhance the success rates of anti-cancer therapies. We show that targeting MARCO works well together with currently used immunotherapies anti-PD-1 and anti-PD-L1 Ab resulting in further reduction of tumor sizes. Altogether, this study suggests MARCO directed immunotherapy as new promising immunotherapeutic approach for cancer immunotherapy activating two different immune cells simultaneously to enhance their anti-tumor properties.

### 2.2.4 Antibody targeting of tumor associated macrophages in pancreatic cancer remodels the tumor microenvironment and revives immune targeting of tumor cells (paper IV)

Pancreatic cancer is one of the leading causes of death worldwide with very poor prognosis and is still considered largely incurable. Patients get diagnosed too late and the cancer has mostly already progressed to the point where surgical removal is impossible. Conventional anti-cancer therapies do not work and the immune checkpoint inhibitors anti-PD-1 and anti-CTLA-4 did not show the hoped success. The most common type of pancreatic cancer is pancreatic ductal adenocarcinoma (PDAC; 95% of all cases) where the tumor tissue consists of very dense fibrotic connective tissue. This makes it difficult to deliver drugs into the tumor and low T cell infiltration defines it as "cold" tumor which impedes immunotherapy as well. As shown in paper II and III, we detected $MARCO^+$ TAMs not only in murine tumors but also in human cancers, such as melanoma and breast cancer patients and it has also been found in human lung cancer [179]. Thus, we evaluated MARCO expression also in human pancreatic cancer patients using TCGA database. Interestingly, MARCO was higher expressed in PDAC patients compared to healthy individuals and correlated with less survival. Immunofluorescence analysis confirmed MARCO to be expressed on $CD68^+$ macrophages and we hypothesized that we can also target $MARCO^+$ TAMs in human pancreatic cancer patients.

To study scavenger receptor MARCO in humans in more detail we set up an *in vitro* system. First, we differentiated $CD14^+$ myeloid cells with M-CSF to macrophages before we polarized them with different cytokine cocktails to uncover what drives the highest MARCO expression. We used the known stimuli for pro-inflammatory macrophages (IFN-γ + LPS) and anti-inflammatory macrophages (IL-4 + IL-10) as well as combinations of other immune suppressive cytokines that are known to be present in TME, such as IL-13 and TGF- β. IL-10 alone induced MARCO and CD163 expression, but not CD206 which is a marker for functional immune suppressive macrophages in humans. As the combination of IL-4 + IL-10 induced MARCO, CD163 and CD206 expression, we continued with this cytokine stimulation mix for further experiments. So far not much is known about $MARCO^+$ cells in humans and we found that MARCO correlates with an immune regulatory and tissue remodeling macrophage type in humans with similar gene expression signature as mice, such as increased levels of *il10* and *fizz1*. This verifies our hypothesis that MARCO is a marker for immune suppressive macrophage subtype also in humans.

To evaluate the role of MARCO in human pancreatic tumors, we co-cultured naïve macrophages with different pancreatic cancer cell lines and analyzed phenotype and function of the macrophages. The co-cultures were performed in trans well plates to only allow soluble factors to pass through the trans well inserts. Interestingly, all pancreatic cancer cell lines induced MARCO and CD163 expression but not CD206 - the same phenotype that we observed with only IL-10 stimulation in the previous experiment. Thus, we confirmed the presence of IL-10 in the tumor-conditioned medium which levels were correlating with MARCO expression and neutralizing IL-10 reduced MARCO expression. IL-10 is a known negative modulator of inflammatory responses in macrophages through STAT3 and indeed,

inhibiting STAT3 resulted in decreased MARCO expression suggesting STAT3 is required to induce MARCO.

All tumors comprise of necrotic and hypoxic areas. As hypoxia can cause macrophage polarization towards the immune suppressive phenotype [282], we wanted to investigate the effect of hypoxia on MARCO expression. For this, myeloid cells were co-cultured in trans wells with the human pancreatic cancer cell line that induced the highest MARCO expression under hypoxic or normoxic conditions. Surprisingly, cells that were cultured under hypoxic conditions showed a phenotype that has been described for MDSCs, including high expression of Arginase-1, less HLADR but no change in VEGF or CD206. These cells were also positive for MARCO and CD163. To elucidate this phenotype, we polarized myeloid cells into MDSCs using the known stimulation mix (GM-CSF + IL-6) which showed exactly the same phenotype as the $MARCO^+$ cells under hypoxic conditions. Thus, we concluded that hypoxia drives MARCO expression also on other immune suppressive myeloid cells not only macrophages.

TAMs and MDSCs are known as the most potent immune suppressive cells in the TME, why we next assessed the ability of $MARCO^+$ myeloid cells to suppress cytotoxic T cell and NK cell function. For this, pro- or anti-inflammatory macrophages or MDSCs were co-cultured with T cells or NK cells and the latter were analyzed for activation (measured by IFN-γ production) and proliferation (measured by Ki67 expression). Interestingly, all $MARCO^+$ myeloid cells inhibited T cell and NK cell activity whereas TAMs were slightly stronger suppressors than MDSCs. Also, myeloid cells that were co-cultured with pancreatic cancer cell lines in a trans well system before being co-cultured with T cells or NK cells were sufficient in suppressing the activation and proliferation of T cells and NK cells. This suggests that all $MARCO^+$ myeloid cells are able to suppress cytotoxic effector cells no matter if MARCO expression is cytokine- or tumor cell- induced.

We previously showed that targeting MARCO by Ab can reprogram immune suppressive TAMs to a pro-inflammatory phenotype revealing MARCO as potential target for cancer immunotherapy (paper II). Thus, we produced anti-human MARCO Ab that has also been described in paper III by immunizing MARCO-deficient mice with human MARCO (hMARCO) protein. These antibody clones were tested for endotoxin contamination and two revealed high levels including the commercially available antibody. As the Fc domain is important for the effector functions of therapeutic antibodies [283] we tested the isotypes of our clones and identified them all as IgG1.

To assess the impact of anti- hMARCO treatment on other immune cells, we co-cultured anti-hMARCO Ab or vehicle pre-retreated pro- or anti-inflammatory macrophages with T cells or NK cells. As expected, anti-inflammatory macrophages suppressed T cell and NK cells activation and proliferation dramatically but adding anti-hMARCO Ab to the cultures reversed this impressively. Moreover, the killing capacity of K562 and different pancreatic cancer cell lines by T and NK cells was augmented drastically when they were co-cultured with anti-hMARCO Ab pre-treated macrophages.

The release of extracellular ATP counts as danger signal and leads to macrophage polarization and a more immunogenic TME [170,284]. We have shown in paper I that engaging MARCO by Abs results in the release of extracellular ATP in murine MARCO$^+$ macrophages *in vitro*. Based on this, we examined if targeting the human receptor shows the same effect and in fact, we observed that targeting hMARCO with our in-house produced Abs also resulted in ATP release by primary macrophages. For further analysis, we continued with one Ab clone that resulted in the highest ATP release and had the lowest endotoxin levels. ATP is a key activator of the NLRP3 and this in turns leads to the release of IL-18 [15]. Therefore, we next investigated the involvement of NLRP3 inflammasome and the release of IL-18 after Ab incubation. In co-cultures of anti-hMARCO Ab pre-treated macrophages with T cells or NK cells, we indeed observed that inhibiting or neutralizing NLRP3 and IL-18 prevented the anti-hMARCO Ab mediated effect to re-activate T or NK cells (measured by IFN-γ production) suggesting that the NLRP3 inflammasome is required downstream for functional signaling of hMARCO in contrast to mice where NLRP3-deficient mice did not reveal any involvement for the anti-tumor effect. Further, we validated that anti-hMARCO Ab also reprograms human immune suppressive MARCO$^+$ myeloid cells to an inflammatory phenotype with increased expression of pro-inflammatory genes, such as *Tnfα* and *Il1β*, and the reduction in anti-inflammatory genes, such as *Mrc1* and *Il10*, which goes in line with our findings in mice.

As described above, STAT3 was identified to positively regulate MARCO expression on myeloid cells. Therefore, we inhibited STAT3 in macrophages prior co-culture with T and NK cells and surprisingly, inhibition of STAT3 re-activated IFN-γ expression in T and NK cells to the same extent as anti-hMARCO Ab did.

Finally, we determined the location of MARCO$^+$ cells in human pancreatic tumor sections in relation to T cells and NK cells. Interestingly, MARCO$^+$ cells were infiltrating the tumors whereas T cells and NK cells were predominantly found outside the tumor separated from MARCO$^+$ cells and confirming the poor infiltrating of lymphocytes into “cold” tumors [285]. Increased numbers of T cells and NK cells correlated negatively with MARCO$^+$ cells.

Overall, the data show that MARCO$^+$ myeloid cells are present in human pancreatic cancers. MARCO is found on human immune suppressive TAMs but also on MDSC-like cells and its expression can be induced by pancreatic cancer cell lines. We can target hMARCO with our in-house produced anti-hMARCO Ab and reprogram human suppressive myeloid cells to restore cytotoxic anti-tumor function of human T cells and NK cells. Therefore, hMARCO-directed immunotherapy is a very promising approach in treating pancreatic cancer patients.

## 2.3 CONCLUSIONS AND FUTURE PERSPECTIVES

A dysregulated immune system is the reason for many diseases, such as autoimmune diseases, and can support the development of others, such as cancer. It is challenging to obtain the perfect balance between pro- and anti-inflammatory responses and immunotherapy became an established approach of treating cancer to release the brakes of the immune system. Immune checkpoint inhibitors, such as anti-PD-1 and anti-CTLA-4 Abs, are already used in the clinics and show impressive results improving survival of cancer patients and reducing primary tumor growth as well as metastases formation. However, not all patients respond to current immune checkpoint inhibitors or other anti-cancer therapies including chemotherapy. Therefore, it is crucial to find novel targets for cancer immunotherapy to further improve its efficacy, possibly in combination with other anti-cancer therapies. As TAMs represent an abundant population in most of the tumors and promote tumor growth in several ways, the focus of this thesis has been to uncover the role of scavenger receptor MARCO in mediating immune responses and in particular, its potential as immunotherapeutic target and the impact of targeting MARCO by monoclonal Abs on tumor growth and the TME.

The main findings of this thesis are the following:

*I.* *Targeting scavenger receptor MARCO on marginal zone macrophages in the spleen reduces antigen transport by marginal zone B cells and modulates the subsequent immune response*

Paper I reveals a novel mechanism of how the interaction between MZMs and MZBs in the spleen can be modulated by targeting scavenger receptor MARCO on MZMs. Engagement of MARCO by monoclonal Abs reduces the expression of CD21 and CD62L on MZBs and impairs Ag shuttling and subsequent humoral immune responses. Moreover, targeting MARCO by Abs leads to the release of extracellular ATP by macrophages. ATP signals through purinergic receptors, such as P2X7, and is known to activate immune cells, inducing inflammasome assembly and the expression of IL-1β and IL-18 [16]. Also, extracellular ATP levels polarize macrophages towards a pro-inflammatory M1 subtype with anti-tumoral properties [170] which is why increased levels are good in the TME to initiate immune responses. Simultaneously, we see an increase in CD39 on MZMs upon anti-MARCO Ab injection. The ectoenzyme CD39 is involved in the conversion from ATP to adenosine which in turn has immune suppressive functions. This most probably deals as modulatory mechanism to prevent hyperactivation of these cells. Adenosine can inhibit the activation of many immune cells including effector cells such as NK cells. Thus, paper I identifies a scavenger receptor with immunomodulatory properties as possible immunotherapeutic target for further approaches. Further experiments need to be performed uncovering the signaling mechanism of MARCO on intracellular level as well as if targeting MARCO also modulates other components of the immune system.

II. *MARCO is a marker for immune suppressive tumor-associated macrophages and targeting by antibodies re-polarizes the macrophages and reduces tumor growth*

In paper II, we identify scavenger receptor MARCO as a marker for the immune suppressive TAM subtype and discover MARCO as a novel target for Ab directed anti-cancer immunotherapy by which we can re-program pro-tumorigenic macrophages towards a pro-inflammatory and tumoricidal phenotype. We verified the success of anti-MARCO Ab mediated anti-cancer effect in three different mouse models and confirmed the expression of MARCO on clinical patient samples of human breast cancer and melanoma patients. The first macrophage- targeted therapies aimed to deplete TAMs completely; however, depleting TAMs by using anti-CSF1 Ab is not the right way to proceed as this removes macrophages from the whole system. They are important sentinels and needed for maintaining the homeostasis and clearing apoptotic cells. Thus, it is definitively better to re-program immune suppressive TAMs to tumoricidal macrophages. For the future it will be important to expand the studies to other cancer types and see if MARCO can even work as a biomarker for some cancers. Furthermore, because MARCO expression is correlated with the most aggressive and metastatic cancer types in humans it is important to translate our findings to see if anti-MARCO Ab therapy also works in humans. In paper I we have shown that targeting MARCO by monoclonal Abs leads to the release of extracellular ATP which is known to polarize macrophages towards a pro-inflammatory phenotype [170]. This goes in line with our findings in paper II, that after anti-MARCO Ab treatment immune suppressive TAMs shift towards a pro-inflammatory phenotype on gene expression and protein level analyzed by flow cytometry. Further studies addressing the exact mechanism of action of scavenger receptor MARCO need to be performed to fully understand how engaging MARCO by Ab leads to reduced tumor growth. More insights are needed to design clinically relevant combinatorial treatments and to reach maximum treatment success.

*III. MARCO engagement on tumor-associated macrophages by antibodies recruits and activates NK cells to increase their TRAIL-dependent tumor cell killing*

Paper III partly elucidates the mechanism through which anti-MARCO Ab treatment leads to reduced tumor growth in the B16 melanoma model. Engagement of MARCO by monoclonal Abs results in metabolic re-programming of the immune suppressive macrophages including increased glycolysis and HIF-1α expression that is known to be upregulated in pro-inflammatory macrophages [281,286]. Furthermore, targeting scavenger receptor MARCO on TAMs activates NK cells to enhance their TRAIL-dependent tumor cell killing. In paper I we have shown that engaging MARCO by monoclonal Abs releases extracellular ATP and, in this study, we observe that the anti-tumor effect is dependent on ATP-sensing receptor P2X7 (P2X7R). P2X7R is known to activate the NLRP3 inflammasome [16] but we could not confirm any dependency on NLRP3 inflammasome in the anti-MARCO Ab mediated anti-tumor effect using NLRP3-deficient mice. Moreover, we discovered that $MARCO^+$ TAMs share perivascular TAM markers TIE2 and CXCR4 that are closely located to $CD31^+$ blood vessel. $TIE2^+$ TAMs are important for tumor vascularization [225,226] and targeting MARCO by Abs also

indicates a reduction in the tortuosity of intra-tumoral $CD31^+$ blood vessels and a trend to less branching of vessels. This further supports our findings in paper II that MARCO engagement results in macrophage repolarization with decreased tumorigenic features. Pre-clinical experiments in the B16 melanoma model revealed anti-MARCO Ab treatment well working in line with other immune checkpoint inhibitors anti-PD-1 and anti-PD-L1 Abs. Current immune checkpoint therapies have their limits and as some of the melanoma patients only have partial responses or do not respond at all we believe that the anti-MARCO Ab therapy can be complementary and thus, hopefully increase survival rates and the number of responders. Combining anti-MARCO Ab with e.g. anti-PD-1 Ab would target three different immune cell types at the same time which makes it a promising approach. Future studies are warranted uncovering the exact mechanism that leads to ATP release in the first place and the role of P2X7R in the metabolic changes and the induction of anti-tumor responses. Additionally, to further increase our understanding of anti-MARCO Ab treatment more experiments are required to evaluate the NK cell dependency in other tumor models.

### *IV. MARCO is expressed in human PDAC tumors and targeting by antibodies re-activates human T cells and NK cells that were repressed by tumor- conditioned medium*

In paper IV we showed that MARCO is expressed on myeloid cells in PDAC patients and it is correlated with less survival. This study goes in line with paper II and III as we also define MARCO to be expressed by immune suppressive cells; however, hypoxia also induces MARCO on MDSCs not only TAMs which is shown for the first time. MDSCs and TAMs are both known as the most immune suppressive cells within the TME and MDSCs can develop into TAMs [287]. In contrast to mice, in humans the anti-MARCO Ab mediated effect on T cell and NK cell activation is dependent on the NLRP3 inflammasome and subsequent IL-18 release. Murine and human MARCO are 68% identical [149], thus it is possible that there are differences in signal transduction. Moreover, human monocytes are much more sensitive for inflammasome activation than mouse cells [288]. We detect that IL-10 induces MARCO expression, while the blocking of IL-10 reduces it. Targeting IL-10 in the TME has been shown to increase anti-tumoral activity by T cells [289]; hence, this might be a possibility to convert the immune suppressive into a pro-inflammatory TME with increased immune cell infiltration and macrophage re-polarization. However, systemic inhibition of IL-10 is not encouraged as this also inhibits its effects in inflammatory diseases where IL-10 deals as important anti-inflammatory modulator. Conventional therapies and T cell-directed immunotherapies have failed to treat pancreatic cancer successfully; thus, the anti-hMARCO Ab treatment is a promising approach to treat this deathly cancer type. As pancreatic cancers count as “cold” tumors with little lymphocyte infiltration [285] it will be important to increase their activity *in vivo* which we achieved with our anti-hMARCO Ab *in vitro*. Further studies need to be implemented addressing differences between mice and humans underlying anti-MARCO Ab treatment, such as the dependency on P2X7R signaling. Also, MARCO targeted immunotherapy needs to be validated in mouse pancreatic cancer models and further in humanized murine models to translate it into humans.

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*"It`s not about the destination, it`s about the journey"*

- *Unknown author*

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